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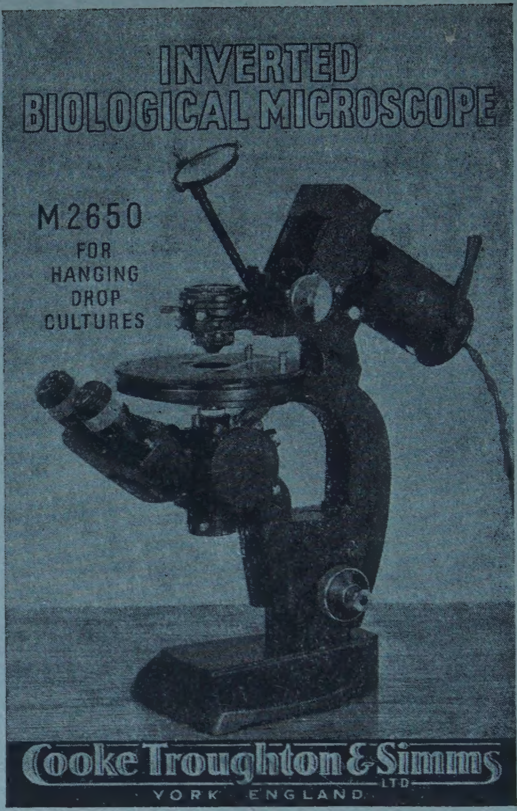

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TURBIDITY AND THE POLARIZED LIGHT ORIENTATION OF THE CRUSTACEAN *MYSIDIUM**

BY RICHARD BAINBRIDGE
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AND TALBOT H. WATERMAN
Department of Zoology, Yale University

(*Received* 31 *January* 1958)

INTRODUCTION

While behavioural responses to polarized light have been demonstrated in many arthropods, relatively little is known about the mechanism of polarized light analysis or its possible application to the navigation of aquatic animals (Waterman, 1958). We have found that the marine mysid, *Mysidium gracile* (Dana), exposed to a vertical beam of linearly polarized light shows preferential orientation by swimming predominantly in directions at right angles to the plane of polarization (Bainbridge & Waterman, 1957). While statistical analysis of the data indicated that this orientation to the *e* vector was significant overall, these crustaceans showed random horizontal orientation 40% of the time on an average. Orientation within 15° of the normal to the polarization plane occupied less than 40% of their time.

This lack of precision in the observed orientation pattern may depend on sensory inaccuracy, behavioural inattentiveness or some unsuspected factor. However, the possibility that the turbidity of the medium might also somehow be involved occurred to us because of the earlier work on *Daphnia*. The quantitative results of Eckert (1953) are not suggestive in this respect, but the observations of Baylor & Smith (1953) are. These authors usually conducted their experiments in water made turbid by the addition of yeast.

Under this condition *Daphnia* and a variety of other cladocerans were reported to swim with a high degree of reliability perpendicular to the *e* vector of a vertical beam of linearly polarized light. A repetition of our previous work on *Mysidium* seemed desirable to test whether or not such high turbidity might affect its polarized light behaviour. Consequently, a series of experiments was designed to compare the mysid's behaviour in carefully clarified sea water and in water deliberately made turbid with yeast.

PROCEDURE

The general experimental method and organism, adult *M. gracile* have been described previously (Bainbridge & Waterman, 1957). However, accuracy and objectivity were increased in the present experiments by photographing the mysid's position every

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* Contribution No. 235 from the Bermuda Biological Station.

5 sec. instead of having an observer manually record the orientation of its antero-posterior axis. A Grass kymograph camera was placed beneath the experimental vessel so that the animal's horizontal orientation was photographed with light provided by the vertical polarized beam itself. Exposures of 0·01 sec. at *f*/8 were appropriate with the Kodak Plus-X film used. Twenty-five pictures were made in each run for a given polarization plane; ten choices from six planes 30° apart and within 180° were tested in random order to comprise a set of runs. Each of such complete groups consisted therefore of 250 orientation measurements and the total data here reported of nearly 4000. In analysing the results the orientation of the animal's longitudinal axis was measured on the photographs relative to the polarization plane; these measurements have been tabulated in nine groups comprising 10° intervals from this plane. 'Clear' and 'turbid' sets of runs were carried out in counterbalanced order. As in previous work a plane cover for the circular experimental vessel eliminated the water-air meniscus. Proper shielding prevented other optical artifacts from originating in reflexions from the vessel's walls.

Clear sea water was prepared at first by careful paper filtration; in later sets it was filtered through a porcelain candle. For comparison, turbidity was produced by adding specific numbers of drops of a standard yeast suspension of about 10 mg./ml. of dried yeast powder. While a suspension of such irregular particles, large relative to the wavelength of the light, is not ideal for causing optically simple and reproducible light scattering (Stacey, 1956), it does have the obvious advantages of being nontoxic, convenient, and of being similar to the suspension used for *Daphnia* by Baylor & Smith (1953).

RESULTS

In all, five paired comparisons were made of clear water *v.* turbid water produced with three to four drops of yeast suspension. In addition two three-way tests were carried out with clear water compared with a moderately turbid medium containing four drops of the standard yeast suspension and with more turbid water containing eight drops of yeast. Each set was done with a different individual *Mysidium*. The resulting distribution of the mysid's horizontal orientation within the nine 10° intervals is shown in Table 1. These figures demonstrate that in clear water the animal's orientation is ordinarily more random than in turbid water. In the latter medium there is usually a distinct tendency to swim perpendicular to the *e* vector, as reported earlier. These generalities become more obvious when all these results are summed as shown in Fig. 1.

The total data show that in clear water the mysids spent on the average 10·9% of their time swimming within 10° of the polarization plane and 11·8% swimming within a similar 10° range at 90° to the *e* vector. The intermediate orientations also show a small, but persistent trend favouring perpendicular orientation. However, the statistical significance is low according to a χ^2-test. By contrast in the turbid media an average of 8·3% of the time was spent within 10° of the plane and 15·1% within 10° of its perpendicular. Furthermore, the overall tendency to orientate at right angles to the *e* vector is here significant at better than the 1% level.

Table 1. *Summary of* Mysidium *orientation data*

Date	Turbidity	Angular groups								
		0–10°	11–20°	21–30°	31–40°	41–50°	51–60°	61–70°	71–80°	81–90°
31 July 1957	Clear	25	25	25	24	24	30	18	17	36
	Turbid (low)	24	16	25	22	24	34	35	30	39
2 Aug. 1957	Clear	31	35	25	26	23	28	29	31	22
	Turbid (low)	20	28	36	18	29	27	34	25	30
5 Aug. 1957	Clear	25	23	24	33	24	25	25	37	32
	Turbid (low)	19	14	17	25	20	26	31	39	57
8 Aug. 1957	Clear	34	21	34	22	27	29	29	26	22
	Turbid (low)	29	22	30	28	22	17	36	29	33
21 Aug. 1957	Clear	25	25	20	30	29	31	24	28	27
	Turbid (low)	25	27	25	19	19	20	36	36	39
22 Aug. 1957	Clear	26	17	21	26	26	27	38	26	35
	Turbid (low)	20	19	29	26	25	27	32	35	34
	Turbid (high)	15	22	22	20	24	34	32	44	33
30 Aug. 1957	Clear	16	23	24	26	30	21	24	33	22
	Turbid (low)	18	25	22	30	38	37	43	35	39
	Turbid (high)	21	21	21	26	23	41	50	43	45
Totals	Clear (No.)	182	169	173	187	183	191	187	198	196
	(%)	*10·9*	*10·1*	*10·4*	*11·2*	*11·0*	*11·5*	*11·2*	*11·9*	*11·8*
	Turbid (No.)	191	194	227	214	224	263	329	316	349
	(%)	*8·3*	*8·4*	*9·8*	*9·3*	*9·7*	*11·4*	*14·3*	*13·7*	*15·1*

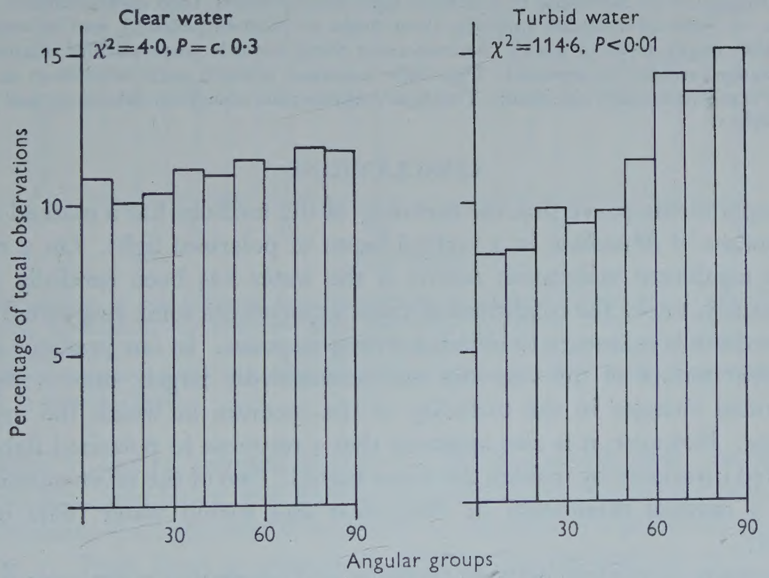

Fig. 1. Comparison of the swimming orientation of *Mysidium* to polarized light during nearly 4000 measurements in filtered water and in water made turbid with suspended yeast. These results show a significant overall tendency to orientation at right angles to the *e* vector in turbid water, but the similar trend in clear water is not statistically significant. This figure displays all the numerical data in Table 1.

If the two sets performed at three turbidity levels (data of 22 and 30 August) are examined separately, an increasing trend towards perpendicular orientation is evident in the measurements for clear, moderately turbid and more turbid media (Fig. 2). But, as in the overall data, the statistical significance is low in the case of clear water and high in the two cases with yeast added. The comparison between the three conditions is not significant when tested for the 10° intervals in a contingency table, probably because *n* is too small, but is significant if 30° intervals are considered.

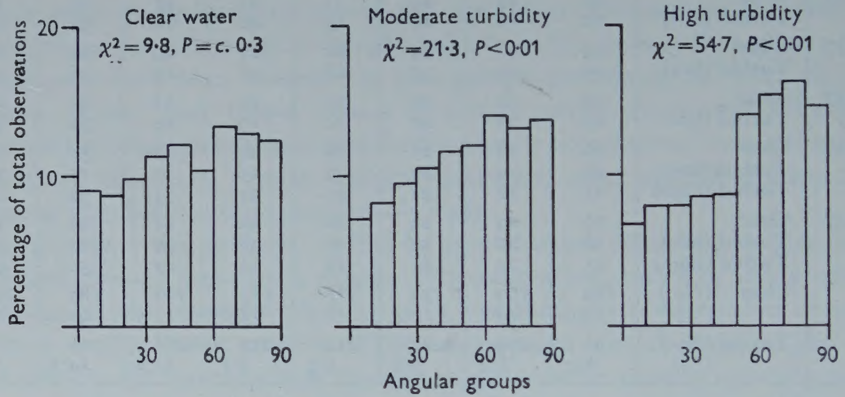

Fig. 2. Orientation of *Mysidium* to polarized light during nearly 1500 measurements in filtered water, in water of moderate turbidity (four drops of yeast suspension), and in water of high turbidity (eight drops of yeast). An increasing trend towards perpendicular orientation with increasing turbidity is apparent. The slight tendency towards such orientation in the clear water is not statistically significant. This figure displays the numerical data for 22 and 30 August in Table 1.

CONCLUSIONS

These experiments prove that the turbidity of the medium has a marked effect on the responses of *Mysidium* in a vertical beam of polarized light. On a statistical basis no significant orientation occurs if the water has been carefully clarified. Consequently, under the conditions of these experiments some suspended material in the medium is necessary to obtain a strong response. In our previous work the inconsistent nature of the response was undoubtedly largely due to slight, but uncontrolled changes in the turbidity of the medium in which the mysid was swimming. However, it is also apparent that a response to polarized light cannot be invoked invariably by making the water turbid. Two of the seven animals tested showed a random orientation in both clear and turbid water (data of 2 and 8 August).

The positive correlation between turbidity and orientation raises some important points relative to the sensory mechanism of this crustacean's response to polarized light. In general, turbidity reduces the polarization by multiple scattering but, more important, the primary scattering of the incident polarized light varies strongly in intensity with the line of sight. More specifically, the intensity of the light originating by Rayleigh scattering is proportional to $\sin^2 \psi$, where ψ is the angle between the *e* vector and the line of sight. In the experimental set-up this means that the horizontally scattered light, visible to the animal against a dark background, will (for primary scattering only) vary from zero when the animal is looking in a direction parallel to the place of polarization, to a maximum when it is looking in a direction at right angles to this. Consequently, whenever there is appreciable turbidity the mysid's horizontal visual field must consist of two dark and two more or less light sectors related to the polarization plane.

If these sectors are detectable by the test organism and if it is phototactic, preferential orientation could arise from intensity discrimination without any visual analysis of the polarized light *per se*. Orientation perpendicular to the plane would be expected of a positively phototactic form provided that this mechanism were operative. Similarly, one should obtain comparable orientation patterns in a vertical beam of unpolarized light if this were reflected horizontally by two diametrical light quadrants separated by intervening dark quadrants. Such a pattern of illumination can be readily obtained by inserting sectors of white paper in the normally completely dark screen surrounding the experimental vessel

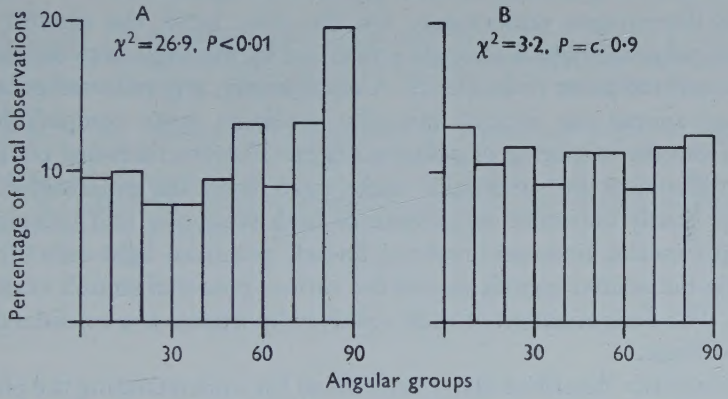

Fig. 3. Orientation of two different *Mysidum* during 250 measurements each in unpolarized light reflected horizontally by alternate light and dark quadrants. One run (B) shows random orientation, the other (A) shows significant preference for swimming in the line of the light quadrants, thus simulating the polarized light response in turbid water. These data are additional to those in Table 1.

This kind of control experiment was run for two sets of data. In one, completely random orientation was recorded; in the other, swimming was strongly directed towards the light sectors as would be expected of a positively phototactic form (Fig. 3). Thus, there is a definite possibility that the intensity of horizontally scattered light is the critical factor in the situation involving a vertical beam of polarized light. But more data are obviously required to make certain of this.

Although our results suggest that *Mysidium*'s response to polarized light under experimental conditions may rest on an external mechanism rather than a specific internal physiological one, the evidence is not entirely conclusive. In this connexion Baylor & Smith (1953) found that black and white sectors in unpolarized light produced orientation in *Daphnia* which simulated their responses to vertical beams of polarized light. Yet they postulated that an analytical mechanism involving reflexion and refraction within this animal's cyclopean compound eye was responsible in the case of polarized light, rather than the external scattering phenomenon which would seem most readily to account for our data.

If the observed orientation of *Mysidium* to polarized light depends on the perception of an intensity pattern rather than of the polarization as such, several important problems are raised. First, it must be determined whether the polarized light responses of other aquatic animals are critically dependent on turbidity. A new series of experiments is currently being done by one of us (T.H.W.) to test how much of the well-marked response of *Daphnia* to polarized light (Baylor & Smith, 1953) remains when it is swimming in very clear water with a minimum of scattering particles. Similar studies need to be made of the various other aquatic forms which respond to polarized light.

The case of terrestrial animals should also be reviewed. While atmospheric turbidity does affect the polarization of the sky (Sekera *et al.* 1955) its influence would probably be less likely to have phototactic significance than in the case of directional illumination underwater. On the other hand, the amount of surface reflexion of polarized light is strongly influenced by the angle between the polarization plane and the plane of incidence. Consequently, any reflected polarized light seen by an animal can include intensity gradients quite comparable to those resulting from the scattering of polarized light. Phototactic forms could use such intensity differences for orientation quite apart from the polarization as such.* Clearly the nearly inevitable occurrence of both scattering and reflexion artifacts in any experimental situation involving linearly polarized light calls for great care in assessing the relative significance of the various potential stimuli available to the organism. The determination of their relative importance is a considerable experimental challenge.

The ambiguities described above are critical for understanding the physiological or ethological mechanism of response to polarized light. But they do not restrict the possibility that animals utilize natural polarized light in the sky or sea. As long as the polarization patterns give rise to any adequate sensory cue detectable by the animal, appropriate orienting or navigational responses could ensue.

SUMMARY

1. The influence of the turbidity of the medium on the previously reported directional orientation of the littoral mysid, *Mysidium gracile*, swimming in a vertical beam of linearly polarized light, has been studied.

2. In carefully clarified sea water the slight preference shown for orientation perpendicular to the polarization plane was not statistically significant.

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* An interesting example of this sort has been described for the phototropic response of the mould, *Phycomyces* (Castle, 1934). Here the growing sporangiophore distinguishes different polarization planes of horizontal light beams by the various intensities of refracted light which penetrate its cylindrical stalk. These vary differentially as a result of changes in the ratio of surface reflexion to refraction.

3. In water made turbid with known amounts of suspended yeast a statistically significant preference for swimming perpendicular to the plane of polarization appeared.

4. This response to the pattern of polarized light illumination appears stronger in highly turbid water than it is in water of moderate turbidity.

5. The mechanism of the observed response seems largely dependent upon discrimination of intensity differences in the light scattered horizontally.

6. These results emphasize the need for careful consideration of the scattering and reflexion artifacts almost invariably present with linearly polarized light.

This research was aided by contracts between the Office of Naval Research (U.S. Navy) and Yale University and between O.N.R. and the Bermuda Biological Station. The authors are grateful to Dr. William H. Sutcliffe, Jr., Director of the Bermuda Biological Station, for his constant helpful cooperation.

REFERENCES

BAINBRIDGE, R. & WATERMAN, T. H. (1957). Polarized light and the orientation of two marine Crustacea. *J. Exp. Biol.* **34**, 342–64.

BAYLOR, E. R. & SMITH, F. E. (1953). The orientation of Cladocera to polarized light. *Amer. Nat.* **87**, 97–101.

CASTLE, E. S. (1934). The phototropic effect of polarized light. *J. gen. Physiol.* **17**, 751–62.

ECKERT, B. (1953). Orientující vliv polarisovaného světla na perloočky (The orienting influence of polarized light on *Daphnia*). *Ceskoslov. Biol.* **2**, 78–83. (Abstract in *Ber. wiss. Biol.* **89**, 198, G. Birukow.)

SEKERA, Z. *et al.* (1955). *Investigation of Polarisation of Skylight*, pp. 66 plus appendices. Final Report, U.C.L.A. Dept. of Meteorology, for Air Force Cambridge Research Center.

STACEY, K. A. (1956). *Light-Scattering in Physical Chemistry*, pp. 230. London: Butterworths.

WATERMAN, T. H. (1958). Polarized light and plankton navigation. In *Perspectives in Marine Biology*. Berkeley: University of California Press. (In the Press.)

THE EFFECT OF TEMPERATURE ON THE WATER-PROOFING MECHANISM OF AN INSECT

By J. W. L. BEAMENT

Agricultural Research Council, Unit of Insect Physiology, Department of Zoology, Cambridge

(*Received* 24 *January* 1958)

INTRODUCTION

Ever since Ramsay (1935) first demonstrated that drops of water placed on the surface of a cockroach were waterproofed by a grease spreading from the cuticle, information has been sought on the role of cuticular lipoid, and on the mechanism of waterproofing. The extension of Ramsay's work to insects in general, stems principally from the researches of Wigglesworth (1945) and Beament (1945). Wigglesworth showed that in a large number of terrestrial species the cuticle contains a waterproofing barrier located superficially in the epicuticle; its lipoid nature was revealed by the effect of solvents and of emulsifiers. Beament demonstrated that much of the waterproofing properties could be simulated by making artificial 'cuticles' using waxes extracted from cast skins; he concluded that the essential part of the waterproofing mechanism was a discrete layer of wax. Subsequently, Wigglesworth (1947, 1948) followed the deposition of the cuticle in *Rhodnius* and *Tenebrio* and showed the relationship of the wax to the other cuticular components. Ramsay (1935) also tested the effect of temperature on the permeability of the cockroach. He suggested that at lower temperatures, the cuticle was impermeable and little affected by temperature, but that—at about 30° C.—there occurred a sharp transformation to a system of high permeability, increasing steeply with temperature. He illustrated the effect by drawing two straight lines through the plot of permeability values at various temperatures, but adduced to a constant saturation deficiency.

Consequently, transpiration/temperature investigations figured largely in Wigglesworth's and Beament's work. As well as the correspondence between such properties for an intact cuticle and for its wax on an artificial membrane, the observations suggested that each species exhibited a 'critical temperature' at which the permeability of the wax increased quite sharply. Beament's measurements of the thickness of wax in cuticle indicated that the permeability was remarkably low, even for such a substance as wax, so he considered that this was due to an organized molecular structure within the wax, which changed at the critical temperature or 'transition point'.

Subsequently, a number of workers—particularly Edney (1951)—pointed out that since the saturation deficiency of dry air increases exponentially with temperature, the rate of water loss from the cuticle might also be expected to change in this way, thus giving a plot with the appearance of a sudden steepening of gradient, but not implying a discontinuous change in the waterproofing mechanism. Later, Holdgate & Seal (1956) made an extensive study of the cuticles of *Rhodnius* and *Tenebrio*. They plotted their results in a way which they suggested would eliminate the effect of increasing temperature on saturation deficiency, and could find little evidence for a sudden breakdown in the waterproofing mechanism at a particular temperature. On the experimental evidence which they presented, their conclusions were certainly justified, and they suggested that the permeability of the cuticle could be explained in terms of diffusion through a membrane with capillaries. However, the author has concluded that the experimental methods on which all the previous workers—himself included—have based their arguments, are so inadequate as to render invalid the interpretations of the structure and the physical chemistry of the waterproofing process based on these methods. For this reason, he has developed the techniques elaborated below, and started a reinvestigation of the problem of cuticle permeability; this paper deals with the permeability of the cuticle of the cockroach, for it was with this insect that Ramsay (1935) made the first investigation in this field.

THE MEASUREMENT OF CUTICLE PERMEABILITY

The term ‘saturation deficiency’ is used to denote the difference between the pressure of water vapour at saturation and that actually obtaining at the same temperature. It will be expressed in mm. Hg and represented by ‘S.D.’. Percentage relative humidity will be represented by ‘R.H.’.

In the original experiments of Wigglesworth (1945) and Beament (1945) water loss was promoted by the simple process of placing a quantity of the drying agent, phosphorous pentoxide, in a large conical flask and suspending the specimen in the air in it. Temperature control was obtained either by immersing the flask in a large water-bath, or by placing it in a crude oven. An ordinary mercury thermometer in the air revealed temperature, and water loss was measured by removing the specimen at intervals to a chemical balance and determining the loss in weight. The authors appreciated that their methods left much to be desired: in particular, experiments were usually severely limited because the duration of desiccation was governed by the water content of the specimen. While small improvements were added by Edney (1951) who used a continuous air stream, and Holdgate (1956) who stirred the air in an enclosed space, subsequent workers have in general adopted this type of experimental method; at best, there has been the virtue that a direct comparison of the results of the various workers is possible.

The physico-chemical controversy which followed, arose out of the interpretation of evidence which purports to show the temperature/permeability relationship of the cuticle membrane. This evidence contains a grossly invalid assumption. All the experimental results so far produced relate to the evaporation from the cuticle at various *air* temperatures; while, ecologically, the evidence is worth its face value as indicating the amount of water which an insect in similar environmental circumstances in the field might be expected to lose, as evidence of cuticle permeability for physico-chemical interpretation it is useless. What is wanted is the relationship of cuticle permeability to *cuticle* temperature, which must necessarily be less than the temperature of the surrounding air.

Consider an insect of 100 mg. suspended in a stream of dry air. Evaporation from it involves a loss of latent heat, and without an external heat source its temperature would fall by 5° C. for every mg. evaporated. However, as soon as it is cooled, heat flows in from the surroundings by conduction and convection. A thermal equilibrium is thus established between evaporative heat loss and heat gain through temperature difference. Since it has been shown (Jeffreys, 1918) that the laws governing molecular conductivity and diffusivity are the same, and since it is clear from the work of Pasquill (1949) that similar relationships hold when one considers eddy diffusivity, it follows that as the permeability of the cuticle increases, and thus the rate of evaporation, so the temperature difference between cuticle and air must increase in strict proportion. Thus (Fig. 1 A) to each evaporation rate, and corresponding air temperature, there is also a cuticle temperature which is less than the former by an amount proportional to the evaporation rate. If we could obtain this proportionality, and work under ideal conditions, the cuticle temperature curve could be derived from the air temperature data; a rough calculation shows that the difference is likely to be of the order of 1° C. when water is evaporating from the insect at 0·1 mg./min. Such differences are therefore not to be ignored.

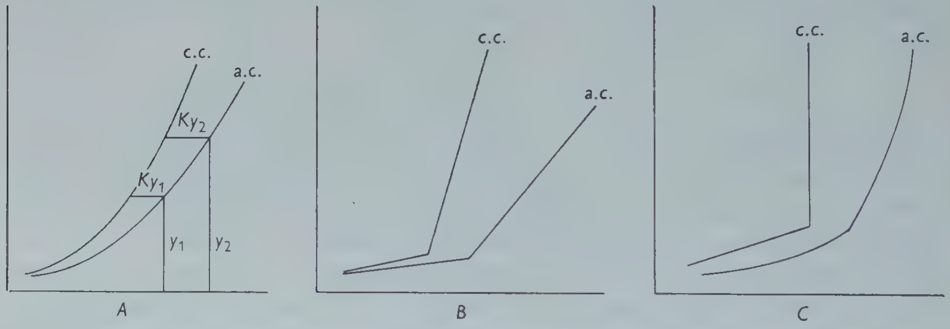

Fig. 1. Possible relationships between the 'air' temperature curve (*a.c.*) of water loss and the 'cuticle' temperature curve (*c.c.*) of water loss. For explanation, see text. Ordinate, rate of water loss; abscissa, temperature.

A knowledge of the temperature of the cuticle is important for other reasons, besides that of assessing the true temperature coefficient of cuticle permeability. It is obvious from Fig. 1 A that if the plot of the rate of evaporation against air temperature (the 'air' curve) is a smooth curve, so also will be the plot of rate of evaporation against cuticle temperature (the 'cuticle' curve), although it will be everywhere steeper than the 'air' curve. Where the 'air' curve shows a discontinuity, however, it is clear from Fig. 1 B that the 'cuticle' curve will also show that discontinuity and will define its position more clearly because of the greater slope.

Indeed, it is even possible that an 'air' curve with a finite slope could correspond to a 'cuticle' curve taking the form of a vertical line (Fig. 1 C).

Turning our attention to the actual experimental methods used by previous workers (see above) several possible sources of error can be suggested, many of which have been demonstrated experimentally by the present author.

(1) Fluctuations of ± 1° C. have been recorded in the air in conical flasks surrounded by a water-bath, while an ordinary mercury thermometer shows no change. The sharpness of a discontinuity will not be revealed by experiments conducted in fluctuating conditions, because each determination will be a mean value for a temperature range.

(2) The equilibrium between an evaporating surface, air, and a drying agent is established very slowly. Thus, for a large part of the time occupied by a typical water loss determination, the S.D. is not maximal and the measured rate of loss will be low. Even so, this method will never give conditions equivalent to putting the insect in dry air. Consequently, previous authors have tended to over-compensate for the change of S.D. with temperature.

(3) To ensure conditions in which it is the membrane which limits the rate of water loss, it is essential to ventilate the surface. With insect cuticle at higher temperatures considerable wind speeds must be used, or the recorded losses will be low.

(4) Apart from the disturbance of stable conditions within the flask when it is opened to withdraw the insect, significant errors arise either from weighing the insect while hot, or from allowing it to cool (and to gain or lose water) before weighing.

Experimental methods

A large programme of technical development has been carried out in order to overcome as fully as possible the objections to previous work, outlined above.

Temperature control. The control of air temperatures, especially in small spaces, is a very difficult proposition and commercially available equipment for this purpose does not appear to exist (Beament, 1958*a*). However, Beament & Machin (1958*a*, *b*) have developed a method which is ideally suited to this purpose. Very briefly, the source of heat which is used is of continuously variable power dissipation, instead of the typical heater which is either 'on' or 'off', and the output is controlled according to requirement by electronic methods. The temperature-sensitive element is a thermistor of minute thermal capacity. Now it is generally true that the best temperature control in an enclosed box is obtained at the site of the thermostat; in this equipment the element is movable and can be placed next to the specimen under investigation. The device also has the useful characteristic that the heater current remains almost constant when control has been achieved, provided that circulation over the element is adequate; but when objects in the box interfere materially with the air circulation the heater current fluctuates appreciably in a cyclical fashion, and this can be used to determine the most satisfactory arrangement of the contents of the box. The device accommodates very rapidly either to changes in thermal loading, or to new temperatures; it is possible to obtain control to ± 1/50° C. over a temperature range 15–35° C. and to ± 1/25° C. over the range 35–55° C. The layout of the equipment is shown in Figs. 2 and 3.

Measurement of air temperature. This was also carried out by the use of a thermistor (Beament & Machin, 1958*a*); the temperature of the air near the specimen can be obtained with a sensitivity at least equal to that of the control element. Information is presented remotely as a reading on a large meter, which can follow quite rapid fluctuations in temperature. The heat dissipation of the sensitive element (an important characteristic of all electrical resistance thermometers) is so small that it is not sensibly affected by the wind-speed over its surface.

Measurement of loss of water. The rate of water loss of an intact dead insect is best measured as rate of weight loss, and for reasons given above the arrangements for weighing should not require either the opening of the box or the transfer of the insect to a balance. The order of weight loss to be detected may be as little as 0·2 mg., so that a delicate weighing method is needed; the specimen may be 500 mg. or more, on the other hand, so that even though a torsion method might be the most convenient way of detecting such small weight changes, it cannot be used directly to give a sensitivity of 1/2500, such as this experiment requires.

The method eventually chosen used a combined beam and torsion principle. The main bulk of the specimen was counterpoised on a beam balance, to which a torsion balance of high sensitivity and smaller range was attached (Figs. 2, 3); thus the loss in weight of the specimen was countered by applying suitable force from the torsion spring to bring the beam back to its original position. The centre of gravity of the loaded beam must be arranged to produce a sensitivity of the same order as that of the torsion balance; this does not mean, however, that the position of the equivalent fulcrum of the beam need be fixed, nor that the suspension should be free of torque. All that matters is that both should be constant at the one chosen null-point position. Both these considerations have very important consequences or the method of measuring cuticular temperature.

Measurement of cuticle temperature. The device for measuring cuticle temperature must not only be comparable in sensitivity with that for measuring air temperature; it must be carried on the beam and it must also be extremely small, to equilibrate with a surface temperature. For these reasons, thermocouples were used. Both junctions were attached to the specimen end of the beam, one arranged conveniently for insertion into the specimen, the other lying alongside the thermistor element measuring air temperature. The current generated in the thermocouple system thus measured the temperature difference between the cuticle and the air, the air temperature being already measured absolutely. The suspension of the beam was made of two parallel nichrome wires, 0·001 in. in diameter, attached to an insulating cross-member at the centre of the beam (Fig. 2) and to similar insulating units in the roof of the box; these wires also served to carry the thermocouple current to a suitable meter.

The circulation of air. The provision of circulating air is neccessary both for temperature control and also for the removal of water vapour from the evaporating

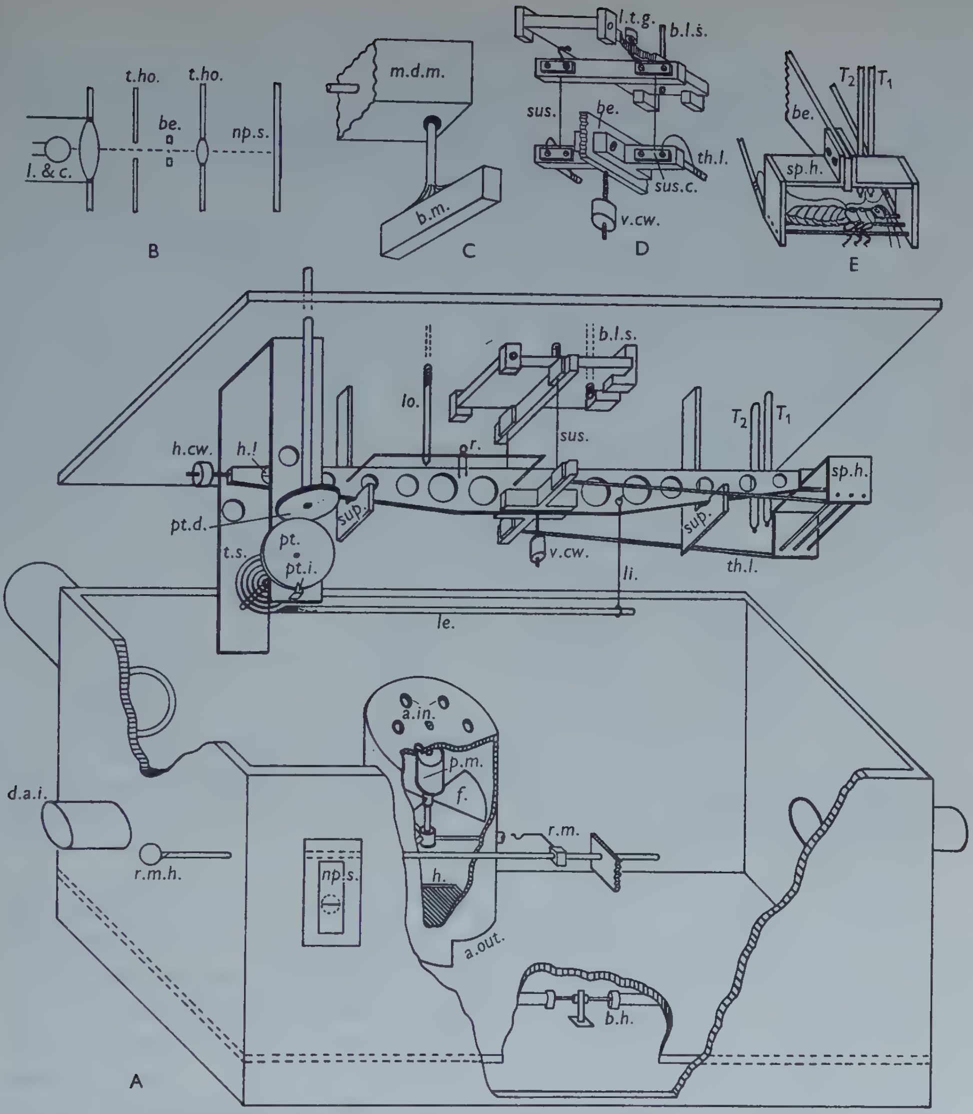

Fig. 2. Detail of equipment described in text; A, orthogonal projection of polystyrene box containing air heating and circulation equipment, with lid raised to show weighing mechanism suspended from it. B, section of optical attachment to project position of beam on to scale. C, magnetic drive for circulating fan, lowered over lid when box is closed. D, projection of beam suspension seen from end of box, showing method of conducting thermocouple current out through suspension. E, projection of specimen holder seen from end of box, showing cockroach in position and thermocouples: one junction under thoracic fold and one junction positioned between the two thermistors. *a.in.* and *a.out.*, air inlet and outlet to heater-circulator unit; *be.*, balance beam; *b.m.*, bar-magnet driving fan; *b.h.*, background heater; *b.l.s.*, beam lowering screw; *d.a.i.*, dry air inlet and outlet; *f.*, fan; *h.*, heater; *h.cw.*, horizontal counterweight; *h.l.*, hair-line index in beam; *l.* & *c.*, lamp and collimator; *le.*, lever between torsion spring and beam; *li.*, link between lever and beam; *lo.*, beam locking screw; *l.t.g.*, lead to galvanometer; *m.d.m.*, mechanism driving magnet; *np.s.*, scale showing projection of beam for null-point; *p.m.*, pot-magnet on fan spindle; *pt.*, protractor recording torsion of hairspring; *pt.i.*, protractor index; *pt.d.*, remotely operated drive on protractor; *r.*, rider; *r.m.*, rider manipulator; *r.m.h.*, rider manipulating handle; *sp.h.*, specimen holder; *sup.*, supports for beam when clamped; *sus.*, fine suspension wires of beam; *sus.c.*, clamp for suspension wires, also connected to galvanometer circuit; T_1 and T_2, air-temperature measuring and controlling thermistors; *th.l.*, thermocouple main leads; *t.ho.*, torsion-spring housing, seen in section; *t.s.*, torsion spring; *v.cw.*, vertical counterweight.

surface, and preferably, the rate of circulation should be variable at will. But the amount of heat emitted by any motor would be far too great for the size of enclosure we are using, and with this in view the small fan in the heater shroud was magnetically coupled to a variable speed motor outside the box.

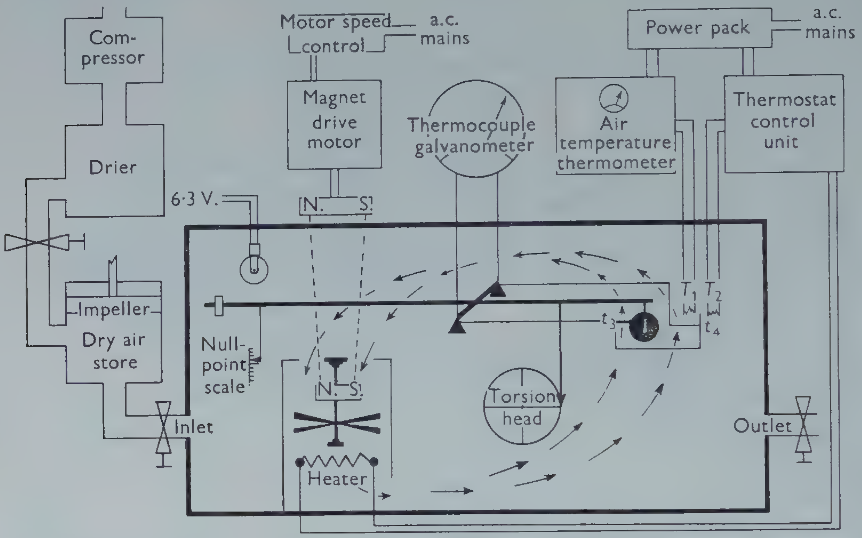

Fig. 3. Schematic drawing of entire equipment. T_1 thermistor measuring air temperature; T_2 thermistor controlling air temperature; t_3 cuticle temperature thermocouple junction; t_4 air temperature thermocouple junction.

Desiccation. Humidity—and especially a fluctuating humidity—cannot be measured accurately (Beament, 1958*b*). Further, Beament (1954) has pointed out that drying agents or saturated aqueous solutions do not give reliable equilibrium atmospheres for ½ hr. or so after any of the many conditions affecting their operation have been disturbed. Because of this, and of preliminary investigations made into the conditions in conical flasks containing a drying agent, a new approach was made. The information needed in these experiments is the actual S.D. of the air in the box. Chemically dried air can be readily obtained, and the actual S.D. can be calculated if one knows the temperature and the amount of water introduced into a known volume of dry air, provided that no adsorptive surface is present. Water vapour is adsorbed on to glass and most other materials, but certain plastics, of which polystyrene is particularly convenient, have no measurable water adsorption even when steeped in liquid water. A vessel of such material is thus ideally suited to enclose a volume of dry air in which a known R.H. can be obtained by admixture of a known quantity of water. Further, a cement of distrene in benzene can be used to coat metals and other components of apparatus inside such a vessel, and when it has dried thoroughly it gives them a similarly non-adsorptive surface.

The impossible aim of maintaining any particular constant humidity inside the box was therefore abandoned; instead, it was proposed to start with the box filled with chemically dried air and to ensure that the only surface exchanging water vapour with the air was the transpiring insect under study. The recorded weight loss of the specimen is in fact the amount of water being added to the air originally dry, so that, provided the volume of the container is known, the actual S.D. at any point in the experiment can readily be calculated.

Construction of equipment. The container (Figs. 2, 3) was a polystyrene box having an internal capacity of 1520 ml. This plastic is, additionally, a remarkably fine thermal insulator, so that few further precautions were needed in this respect. A portion of a second similar box was used to form an air-jacket round the bottom of the main container, and this space included a number of carbon resistors, such that it was possible to dissipate about 5 W. 'background' heat for work at higher temperatures. Leads to the heater element were brought through air-tight seals in the base of the box; the outlet from the fan-heater shroud was aimed to provide the most efficient circulation over the specimen and the three temperature-sensitive elements. Non-magnetic materials were used throughout to avoid any interaction with the magnetic field driving the fan. The balance beam was of Elektron metal and extensively lightened by perforation; its suspension and the whole of the accessory mechanism associated with weighing were attached to the lid of the box, which was consequently strengthened by brass girders to prevent distortion when hot. To support the specimen, a grid of phosphor-bronze wires was constructed, thus to provide for the maximum of air circulation. This specimen holder was also strengthened by bronze rods running to the centre of the beam, and these rods carried the thermocouple current to the suspension. The thermocouples were of 44 s.w.g. copper-constantan, pot-brazed with silver-cadmium solder. All parts of the thermocouple circuit, except for the junctions, were heavily coated with distrene cement to prevent local heat conduction through the wires.

The suspension was attached to the lid of the box so that the unit clamping the upper ends of the nichrome wires could be raised and lowered by a screw passing through the lid; thus the whole beam could be raised to swing free, or lowered on to two V-rests, one at each end, in which position it could be clamped by a further screw. The specimen could thus be mounted without risk to the fine suspension. The other end of the beam carried an adjustable counterweight and a graduated slide for small 'rider' weights; these latter could be manipulated by a lever passing out through the side of the box. Below this end of the beam also, but attached to the lid, a plastic cage carried a coiled hair-spring, from which a fine glass capillary applied tension through a pivot-link to the 'specimen side' of the beam. The shaft bearing the torsion spring also carried a 360° graduated protractor, from which the degree of torsion applied by rotating the shaft could be obtained; rotation was transmitted by a friction-drive wheel on a vertical shaft through the lid of the box.

A horizontal cross-wire was mounted in one of the holes in the beam, and an image of this was projected on to a graduated screen on the side of the box; this allowed the accurate location of the beam in its zero position during an experiment. The beam also carried adjusting screws, in order that it could be set to appropriate sensitivity. With the exception of the wire of the heater element, the torsion spring and the balance suspension wires, all materials which were not polystyrene were covered with distrene cement.

Plastic tubes were let into the ends of the box, each with sealing devices, so that the enclosure could be flushed through with dry air; the lid was carefully ground to fit the seating on the box, and a small quantity of silicone grease used as a seal. Finally, a minute capillary was left open to the atmosphere to allow for the escape of air during sealing the box, and for thermal expansion (see p. 503).

Calibration. The torsion balance was calibrated against standard weights and riders; in the model used in these experiments, it was arranged that 10° rotation of the torsion spring corresponded to 1 mg. weight change at the specimen. The thermocouples were calibrated in the usual way against Beckmann thermometers, and the galvanometer gave a deflexion of *ca.* 2 cm./° C. difference.

Experimental procedure

The specimen, a large nymph of *Periplaneta americana*, was very lightly anaesthetized with carbon dioxide, and immediately placed in hydrogen sulphide gas for 20 min.; unless narcotized, this species voids faeces violently when first sensing the poison, usually contaminating its surface, and sometimes even rupturing the hindgut. The dead specimen was temporarily stored over phosphorous pentoxide in a large vessel to bring the surface towards a condition of equilibrium with dry air. A store of air at less than 1% R.H. was obtained by passing air at 15 lb./sq.in. through a long column of silica gel and allowing it to escape slowly through a sinter-glass funnel immersed in 98% sulphuric acid. A large store of such dry air was held at atmospheric pressure in large polythene bags. Where the experiment was to be started appreciably above room temperature, the box was also 'warmed up' at this stage; the temperature regulator was then disconnected and the lid bearing the balance was jacked up; the specimen was placed on the holder and the 'cold' thermocouple junction placed under a meso- or meta-thoracic wing-bud, so as to be in intimate contact with the cuticle surface; great care was exercised not to damage the cuticle. A rough balance was obtained, using the counterweight and heavy riders, but ensuring also that if high rates of water loss were expected, a suitable rider had sufficient traverse to offset the bulk of this loss by moving it during the experiment, for the weight loss which the torsion spring could accommodate was limited. The lid was then lowered into the enclosure, the beam was allowed to swing completely freely, and a rider was manipulated to such a position that the image of the cross-wire came to a convenient scale position, with a full traverse of the torsion spring available.

The box was then swept through with a sufficient volume of dry air to replace its previous atmosphere. From this point onwards it is essential to record the total weight loss of the specimen, whether the box is at constant temperature or changing to a new temperature, for until it is reflushed with dry air, *all* losses contribute to the humidity of the enclosed air. The box was then sealed, its air temperature was brought to the required value, and readings were taken at intervals of air temperature, cuticle-air temperature difference and weight loss of the specimen. The box was then brought to a new temperature and the procedure was repeated. According to the temperature excursion and the duration of the experiment, limits are set to the permissible amount of evaporation into the air contained in the box; immediately following a selected reading for water loss, the box was reflushed with air and a further measurement of evaporation rate taken *at the same temperature* as the previous one. The ratio of the two readings at one temperature thus obtained should be the ratio of the two S.D.'s respectively obtaining, which gives a useful check on the experimental procedure. It was found that, providing one was not working close to the maximum temperature obtainable (i.e. with the heater at capacity load) the polystyrene insulation of the box kept it and its contents so well insulated that the heater was easily capable of bringing freshly introduced air up to the required temperature almost at once and closely controlled temperature conditions were restored in a matter of seconds after the box was resealed.

One experimental problem remains: air expands on heating, and to allow for this, a fine capillary leak was provided. But the assessment of the S.D. at any point in the experiment depends on a knowledge of the quantity of moisture evaporated into a known volume of air. In the first place, the experiments with one filling of air were always carried out at a constant temperature, or with increasing temperatures, so that any leakage would always be outwards; thus humid air from the ambient would not enter the box. Since it was found experimentally that there was always an increased rate of evaporation at higher temperatures, frequent air replacement was necessary at higher temperatures, whereas low temperature experiments had to be run for much longer periods in order to get measurable water loss. This circumstance in itself helps to keep down the error involved. In practice the greatest excursion of temperature with one filling of air was well under 10° C., and heating through this interval would cause a volume expansion of 3%. Suppose a maximum of 3 mg. water is allowed to evaporate before the air is changed; in the air volume under consideration, this water would be distributed at 2 mg./l., and over the experimental range we are considering 2 mg./l. is equivalent to 2 mm. Hg vapour pressure. Suppose in the course of one run the air is heated from 25 to 35° C.; the S.D. of dry air at 35° C. is 42 mm. Hg which by evaporation from the specimen has become $42-2=40$ mm. Hg provided no loss has occurred. With 3% of leakage, the actual vapour pressure due to evaporation might be only 97% of 2 mm. so that the error in calculating the actual S.D. at 35° C. is actually 3% of 2 in 40, i.e. 0·2% which is, of course, negligible.

RESULTS

In Fig. 4 the actual water loss in mg. from a large cockroach nymph is plotted against the air temperature and against the corresponding cuticle temperature. The extent of the temperature difference revealed by the thermocouples is thus the horizontal intercept between the curves. The wind speed was constant throughout. Thus the 'air' curve is closely comparable with the curves obtained by previous workers, for in the present experiments the R.H. has not exceeded 10%, and previous workers would have been fortunate to have achieved as close control as this in their experiments using drying agents. The temperature scale is an open one, but no amount of compression of this axis would give the curve any appearance other than of a smooth change of gradient in which no sudden break can be detected. The absence of any discontinuity therefore upholds the objections of Holdgate & Seal (1956).

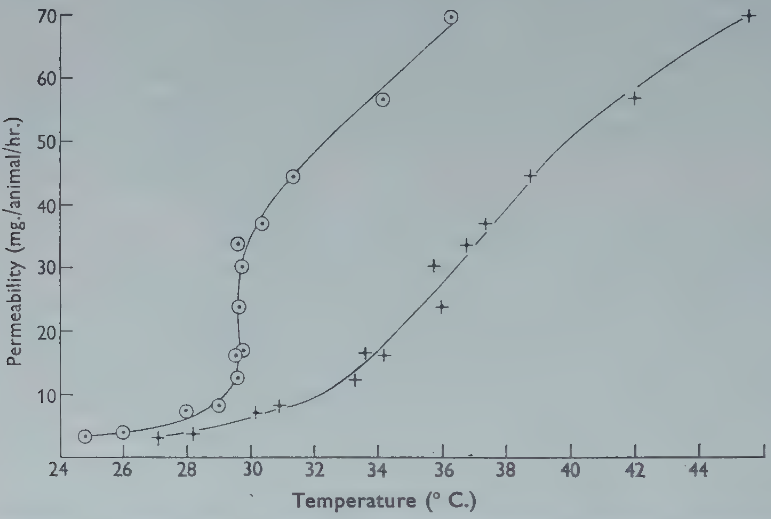

Fig. 4. Graph showing water loss of a large cockroach nymph about 1 week after moulting at various temperatures. +, air temperature; ⊙, cuticle temperature. Humidity (known accurately) varying between 0 and 10% R.H.

However, when the same readings of rate of evaporation are plotted against cuticle temperature, an entirely different picture is obtained. There is a slow gradual increase in the rate of evaporation up to a temperature of about 29·7° C above which the rate of evaporation increases steeply, while the cuticle temperature remains almost constant. Then the temperature of the cuticle again increases with increased rate of evaporation, but with an ever increasing gap between the cuticle and air temperatures. It is immediately obvious that a sharply defined phenomenon in the 'cuticle' curve is completely masked in the 'air' curve.

In order to remove the effect of a changing S.D., due to the changing temperature of 'dry air', Holdgate & Seal (1956) plotted the logarithm of transpiration against the reciprocal of absolute temperature; they thus assumed that in their experiments S.D. increased exponentially with temperature. But the data at our disposal enables us to calculate the actual mean S.D. obtaining at each determination of water loss. Now, under conditions where the permeability of the membrane is limiting, the actual amount of water passing through the cuticle at any temperature will be proportional to the permeability of the membrane at that temperature and to the S.D. Thus, if we divide each determination by the corresponding mean S.D. of the air, we obtain the permeability per unit of S.D. This transformation has been carried out on the data of Fig. 4 to give Fig. 5. This curve gives the true relationship between permeability and temperature, and has the great advantage that it presents the data on a linear scale.

The 'air' curve in Fig. 5 does not give any real evidence of a discontinuity either; when we examine the 'cuticle' curve, however, we find a clearly delineated phenomenon. Below 29·7° C. we have a membrane with a finite temperature coefficient of permeability; above that temperature, although the permeability is greatly increased, there is a similar temperature coefficient. When the membrane is at 29·7° C. the distribution of points justifies only the drawing of a vertical line, the simplest interpretation of which is that the permeability of the cuticle has increased suddenly. It must be realized that the vertical portion of the curve is 'real' (i.e. points have been determined along its length). Over those portions of the curve with finite slope, we know the removal of water vapour to be adequate, so that the limiting factor is the permeability of the cuticle. Over the vertical portion it is still true that ventilation is adequate, but the rate of loss we record is here limited by the rate of heat transfer from the air to the cuticle—a process which determines the amount of latent heat available for evaporation.

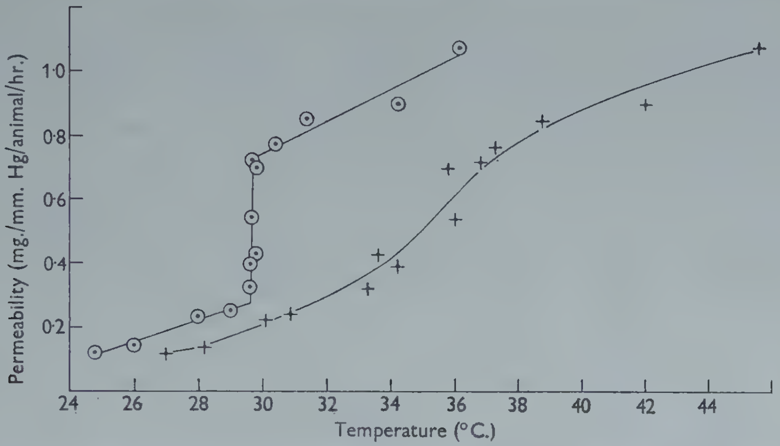

Fig. 5. Graph showing results of Fig. 4 corrected to water loss per unit saturation deficiency of the air at each experimental determination. Symbols as in Fig. 4.

The significance of Fig. 5 will be discussed in more detail after the properties of isolated grease films (p. 507) have been described. It is perhaps worth noting that the particular experiment described above was the last of the series reported in this paper; it involved such high air temperatures as to cause certain damage to the equipment, and though it was essential to obtain such information, the danger was foreseen, and for this reason all previous experiments (which are reported below) were discontinued before the higher temperatures were reached.

It is clear, then, that the cuticle of the cockroach goes through a transition at a sharply defined temperature, at which its permeability increases suddenly. Though previous workers are mostly agreed that the cuticular lipoids are responsible for waterproofing, it would be unwise, in view of the unreliability of their experimental methods, immediately to attribute the transition to any one part of such a complex system as the cuticle; yet it would be surprising if it proved to be the result of interaction of a large number of components, and if we can show a change in one component at the temperature in question it is virtually certain that that component is responsible for the change in the permeability of the cuticle.

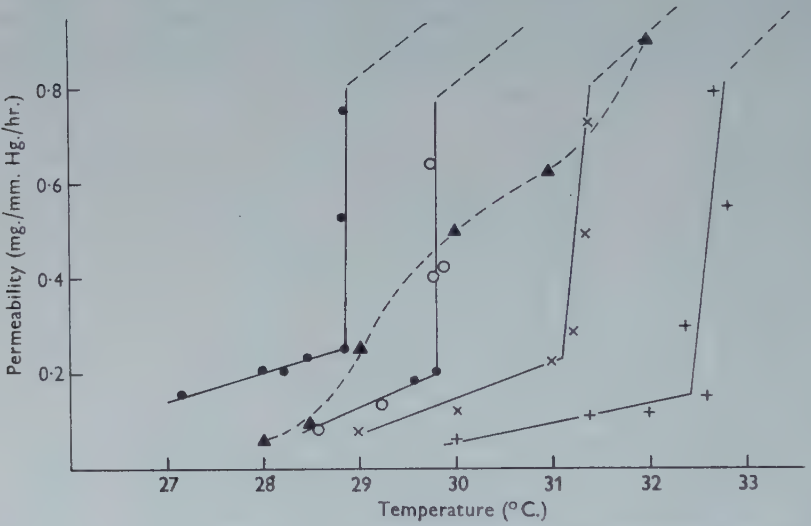

Fig. 6. Graph showing permeability of the cuticle of large cockroach nymphs of various ages within the instar at various cuticle temperatures.
●, nymph 2–3 days after moulting; ○, nymph 1 week after moulting; ×, nymph 2 weeks after moulting; +, nymph 2–3 days before next moult; ▲, and broken line, mean permeability of all four insects considered as a homogeneous sample.

Now Ramsay (1935), in the original investigation, suggested that the temperature of breakdown in waterproofing was around 30° C. If we were to plot the 'air' curve, uncorrected for S.D. on an abscissa with a more compressed scale, and draw two straight lines through the points as he did, the intersection would certainly lie above 29·7° C. Ramsay drew two straight lines to indicate a change of conditions and certainly not to imply two linear functions. Nevertheless, it is important, for many reasons, to establish the uniqueness or otherwise of 29·7° C. for the species *P. americana*. If it is not unique to the species, experiments with a group of specimens which yield only average results could mask the sharpness of a phenomenon nevertheless clearly defined in each. Wigglesworth (1945) showed that the rate of water loss for *Rhodnius* nymphs was very constant for any one temperature, and Beament (1946, 1951) pointed out the sharp collapse at a fairly constant temperature of eggs heated in hypertonic solutions. On the other hand, Holdgate & Seal (1956) showed a change in the temperature characteristics of waterproofing with the age of *Rhodnius* and *Tenebrio* nymphs within the instar. In Fig. 6 the permeabilities of four large nymphs, considered individually, are displayed; the individuals were of the same order of size, but form a series from about 48 hr. after a moult, to within 2–3 days of the next ecdysis. (It is difficult to predict, even with most closely controlled culture conditions, the time of moulting of a cockroach nymph, though it can be estimated roughly by examining the wing buds.) It will be seen that while the form of the curve is similar in all these nymphs, the transition temperature advances with the age of the individual within the instar, by as much as 5° C. The dotted curve shows the mean reading which would be obtained from the four individuals together and although an experimenter would be unlucky (or unwise) to use quite so heterogeneous a group, the necessity for conducting experiments on individuals is clearly shown.

On the other hand, when cockroaches all of approximately the same age within the moulting cycle are compared, their transition temperatures would seem to agree more closely. Thus for five animals, each about 48 hr. after moult, the transition temperature lies between 28·2 and 30° C. When five nymphs, each believed to be within 2–3 days of the next moult are considered, the transition temperatures lie between 33·2 and 36·1° C.; the variation here is greater than for animals at the beginning of the instar. It is clear that the cuticles are more uniform in respect of the temperature-sensitive characteristic of waterproofing when first formed, but that this characteristic may change during the instar.

EXPERIMENTS WITH WATER DROPS

As is well known (Ramsay, 1935; Beament, 1955, etc.,) a drop of water placed on the cuticle of a cockroach is waterproofed against evaporation by the spreading of the cuticular grease over it. The grease spreads so rapidly that one can certainly rely on complete coverage over the surface of a drop of 1 mm.^{3} within 5 sec. of applying it. It is important to compare the temperature characteristics of water loss of the intact animal, as demonstrated above, with those of droplets covered with a film of grease, since a close parallel in the two cases would provide the strongest evidence for the function of the grease on the intact cuticle. It is not, however, at all easy to make measurements on drops placed on the surface of cuticle. Water loss cannot be detected by weighing, for one cannot distinguish between the loss from the drop and from the animal. The drop has a surface of contact with the animal through which water exchange can take place as the author has found in another connexion. Experiments of this kind are therefore much more satisfactorily performed on an artificial surface. The conditions for success are fairly critical. Beament (1955) has already outlined some crude experiments of this kind. Briefly, it is necessary to have a surface with a contact angle of 90° or greater to pure water, otherwise the water spreads out into a thin film when the grease is applied to it, presumably because of the lower 'surface tension' of the combined surface.

Now it is quite simple to measure by thermocouples the temperature difference between a small drop of water and the surrounding air while evaporation is taking place. It is not at all satisfactory to do the same thing when the drop is covered with grease, for the following reasons. When one measures the rate of evaporation from the surface of an intact cockroach one assumes with some justification that the surface area of the animal remains constant, at least for about 5% loss of its contained water. Estimation of the surface area of the animal is extremely difficult, but the form of the curves here presented is not altered by expression in units of surface area, and provided that only single animals are used in each determination it is perfectly satisfactory to express the recorded water loss in mg./animal. But when a water drop—particularly a small one—is placed on a surface and proceeds to evaporate, its exposed surface area changes substantially; even its initial surface area depends on its surface tension (including the effect of any grease film), its size and the nature of the surface on which it is resting. By inserting an object, such as a thermocouple, into the drop the surface is deformed, and even neglecting the difficult technical problem of ensuring that the probe does not interfere with the 'waterproofedness' of the film and that water does not creep up the probe and evaporate from its surface, the determination of the exposed surface area is made excessively difficult if it is of complex shape.

In fact, the form of the profile of a large drop of liquid on a surface cannot be evaluated by rational mathematics (Rayleigh, 1896). If the drop is sufficiently small it may be possible to assume that it takes the form of a cap of a sphere, in which case the calculation of its exposed surface area from simply measured parameters is a straightforward matter.

Experimental method

If a drop, under suitable conditions of illumination, is placed on a horizontal surface and observed from a direction in the plane of the surface through a microscope with a micrometer eyepiece, it is easy to measure both the maximal height and the diameter of the plane of contact of the drop. With a 360° protractor in the eyepiece (in these experiments one was placed in one of the eyepieces of a binocular microscope) and a fixed reference index, the angle of contact of the drop to the surface can also be obtained. A variety of surfaces of different contact angles to water were enclosed in a transparent box, suitably illuminated. The humidity in the box was maintained at about 96% R.H. to keep evaporation to negligible proportions. Water drops of various sizes were applied to the surfaces by an 'Agla' micrometer syringe, and the parameters—maximum height (a), radius of the plane of contact (b) and angle of contact (ϕ)—were measured. The volume (V) of the drop was calculated from the formula:

$$V=\pi\tfrac{1}{6}a\,(a^2+3b^2).$$

The calculated volumes were compared with those measured by delivery of the syringe. The agreement was better than 3% provided the drop was of 1 mm.^{3} or less, though if it is below 0·1 mm.^{3} the parameters cannot be measured sufficiently accurately. It is of interest to note that a drop of 1 mm.^{3} which weighs 1 mg. cannot readily be weighed to 3% and certainly cannot be so weighed if one is trying to detect 0·03 mg. in the combined weight of the drop and a supporting surface of several mg. weight. It was therefore concluded that such optical methods would give the most accurate means of assessing the rate of evaporation from drops. The evaporating surface area (A) is given by

$$A=\pi\,(a^2+b^2)$$

and the contact angle by

$$\sin\phi=\frac{2ab}{a^2+b^2}.$$

The observed contact angle was used as a quick check on the measurements, but since the assessment of contact angles by placing a tangent (methods of Beament, 1944; Holdgate, 1955) is a subjective measurement liable to considerable inaccuracy, calculation by this formula is likely to be more accurate.

For measurement of evaporation through grease films on a water surface a temperature-controlled plastic box as described above was used, but with the balance arm replaced by a specimen holder on a universal joint. Plastic sleeves were let into the lid of the box, through which a micrometer syringe cannula and a needle holder could be respectively inserted, in order to deposit drops of water on to the specimen holder and to add grease to them. A clean thick sheet of polystyrene was placed horizontally on the holder, and a binocular microscope with one micrometer squared eyepiece and one micrometer protractor eyepiece was lined up on it. The temperature was allowed to become stable, the box was swept with dry air, and two drops of distilled water, each 1 mm.^{3}, were deposited rapidly approximately 1 cm. apart, the line joining their centres being at right angles to the direction of flow of the circulating air. The needle, which had previously been stroked over the surface of a large cockroach nymph, within 1 week of having moulted, was rapidly touched against the surface of one of the drops. The necessary parameters of the drops were recorded at suitable intervals of time. The procedure was then repeated at a higher temperature using a fresh surface of polystyrene. As polystyrene is an extremely poor conductor of heat, a considerable time must elapse before the surface to which the drops are applied is at air temperature, and of course it is essential to use a clean surface for each recording. It may be added that the reason for having the cannula in the box is so that the water delivered to the plate shall be initially at the same temperature as the air and the surface. The curve for evaporation per unit surface area against air temperature obtained in this way was of smooth 'exponential' type, and in view of the arguments put forward earlier it was suspected that it suffered from the disadvantages of all the earlier work—in this case, that the polystyrene was such a good heat insulator that the temperature of the drop was well below that of the air. Experiments with unwaterproofed drops and thermocouples confirmed this. But if drops of clean water were placed on a large sheet of a good conductor, such as aluminium, measurements with thermocouples indicated that during evaporation the water temperature fell by only a small part of one degree—the drop can obtain heat from its surface in contact with the metal, and the metal is not only a very good conductor but it also has such a large surface exposed to the surrounding air that it can easily make good the heat lost to the drop. Unfortunately, a metal is readily wetted by water, and cannot therefore be used for experiments with waterproofed drops. It was found that a suitable surface could be obtained by coating the metal with a minutely thin film of silicones, using the solution D.C. 1107 and subsequently baking it. The film was so thin (it is probably of molecular dimensions) that it did not appear to interfere with the transmission of heat from the plate to the drop.

The rates of evaporation per unit surface area are plotted against temperature in Fig. 7, in which the selected data are based on the observed rates of evaporation between the volume limits of 0·95 and 0·75 mm.^{3}, for drops initially of 1·0 mm.^{3}. This precaution kept down the effect of evaporation on the S.D. of the enclosure, and, more important, to some extent limited the suspected effects of ageing of the film with exposure. In view of the individual differences in nymphs already demonstrated, all the data for any one graph was obtained with grease from one nymph; for Figs. 7 and 8 the animal was about 7 days post-moulting. The airspeed was held constant throughout the experiments and, though adequate, was slow, in order to prevent vibration and distortion of the droplet profile.

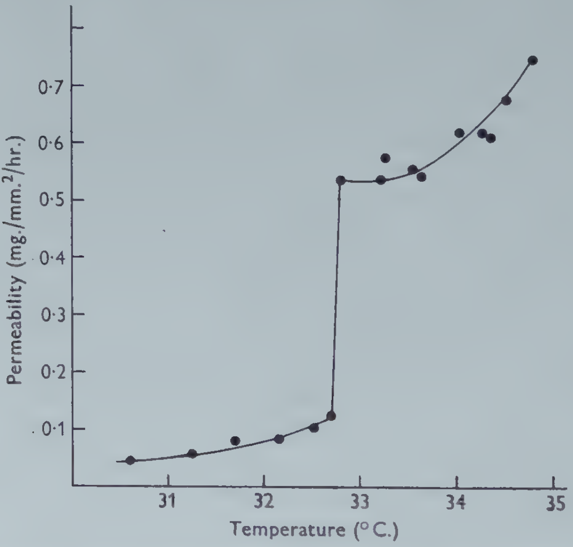

Fig. 7. Graph showing rate of evaporation of water through a thin film of cockroach cuticular grease plotted against temperature, when evaporating system is maintained at air temperature. Humidity between 0 and 5% R.H.

From Fig. 7 it is at once clear that a sudden breakdown in waterproofing occurs; in the experiment illustrated, this takes place at 32·7° C. The observations parallel closely the corresponding measurements on the intact animal (Fig. 4), while Fig. 8 (for permeability per unit S.D.) can be compared with Fig. 5. The permeability does not appear to be temperature-dependent below its transition, but it is probably significantly so above it; the consequences of this are outlined in the Discussion. Two obvious differences are seen: with the whole animal, the vertical part of the curve is 'real' with several determinations along it; the isolated film changes from one permeability characteristic to another and by drawing a vertical line between the two one does not imply that one could determine readings on it—in fact one failed to do so. Secondly, the relative permeability increase at the transition temperature is considerably greater for the isolated film. The vertical part of Fig. 5 is determined by rate of heat transfer, as explained above. But here, where we are dealing with a simpler physical system in which temperature is not being determined by evaporation but is being kept close to that of the air, we should expect the transition to be so represented.

The fact that the two phenomena are so strikingly similar can only mean that, of all the many components in cuticle, the grease is the significant factor limiting water loss.

DISCUSSION

While experiments with artificially waxed membranes (Beament, 1945) provided evidence for believing that the impermeability of insect cuticle is due to an intact layer of freely extractable wax, these models have not been entirely satisfactory for explaining the very high order of impermeability of the cuticle in so far as (*a*) that they have never achieved quite the impermeability of their corresponding natural cuticles, and (*b*) that they have been, like the cuticle, the centre of much controversy over physico-chemical interpretations. The discrete film of grease on water, which from Beament's (1955) study of the composition of cockroach grease seems likely to be of the order of one molecule thick, is in every sense a more satisfactory model; it is likely, in the absence of dust, to be a uniform and completely imperforate layer.

In order to compare directly the properties of the film on water with those of the thick grease on the animal, it is desirable to reduce the observations to common units of absolute permeability, and those selected by Barrer (1941) will be used. The values which we now derive are based on the means of several careful determinations: for the film on water, the measured permeability at 25° C. is quite consistent, and the accuracy of the absolute permeability is therefore higher than that derived for the thick grease on whole animals, since not only have we to contend with typical biological variations but also we have to assume that the thickness of grease on the animals is uniform and we have to estimate the surface area of the nymph.

The mean permeability of the grease film at 25° C. is 2·5 mg./cm.^{2}/hr. which gives an absolute permeability of $0{\cdot}40 \times 10^{-8}$ ml. water vapour at N.T.P./sec./cm.^{2}/mm. Hg/mm. thickness. In this calculation it is assumed (Beament, 1955) that the film will be of the order of 100 Å thick, i.e. one average molecular length. The absolute permeability of the film is thus extremely low compared with any of the substances cited by Barrer (1941). The average permeability of large cockroach nymphs at 25° C. is 3·5 mg./animal/hr. which gives an absolute permeability of $1{\cdot}65 \times 10^{-8}$ absolute units. For this calculation we have assumed that the nymph has a surface area of 7·5 cm.^{2} and a thickness of grease of $0{\cdot}25\mu$. This is still below the value of absolute permeability of the most impermeable natural materials described in the literature; Taylor, Hermann & Kemp (1936) give $2-2{\cdot}1 \times 10^{-8}$ for measurements on slabs of wax, with which our figures can be compared.

Let us then propose a structure for the grease layer and suggest a physico-chemical change at the transition point, and let us subject our hypothesis to a number of tests based on information we can calculate from the absolute permeabilities and from the graphs above. We suggest that, below the transition temperature, the grease layer on the water droplet surface is organized, by the attraction of the polar endings of the long-chain molecules of the grease for the water, to form a solid, tightly packed orientated 'monolayer'. It is this structure which accounts for the very great absolute impermeability of the film. If this is so, then the lowermost portion of the thick grease on the animal, which is also against a wet surface, must also be orientated and organized into a layer similar to the film on water. The remainder of the thick grease, which is without such a polarizing influence, must presumably be a fluid with the typical random lack of organization of the molecules of liquids. Since we know the permeabilities of the organized film and of the film plus the thick grease and since we also know the thickness involved, it is a simple matter to calculate the absolute permeability of the thick unorganized portion alone. From the thicknesses used in the calculations above, the 'monolayer' to thick grease stands in the ratio 1:24. If we convert permeability into resistivity by dividing thickness by permeability we obtain properties which can be added thus

$$\frac{d_1}{p_1}+\frac{d_2}{p_2}=\frac{d_3}{p_3}.$$

The thicknesses (d) are as 1:24; 25; the permeabilities p_1 ($0{\cdot}4\times10^{-8}$) and p_3 ($1{\cdot}65\times10^{-8}$) for film and total grease on the animal can be substituted to find p_2, the absolute permeability of the unorganized fluid grease at 25° C.:

$$\frac{1}{0{\cdot}4}+\frac{24}{p_2}=\frac{25}{1{\cdot}65}\quad\text{(all divided by }10^{-8}\text{)}.$$

From this, p_2 is $1{\cdot}85\times10^{-8}$; it can be compared with Taylor, Herrmann & Kemp's figure of $2-2{\cdot}1\times10^{-8}$ for pieces of wax which also are presumably without molecular organization.

Secondly, we propose that at the transition temperature, the organized structure of the 'monolayer' breaks down—possibly because of the accumulation of thermal energy in its molecules which eventually overcomes the forces attracting the polar groups to the water. All the molecules would then assume the random configuration of a fluid. The absolute permeability of the film on water would thus jump at the transition temperature from that of organized layers to that of the fluid portion of the thick grease which we have just determined (presuming the figures to be of the correct order at transition temperatures as well as at 25° C. which will not involve great errors). The ratio of these two values, $0{\cdot}4\times10^{-8}:1{\cdot}85\times10^{-8}$, gives approximately a fivefold increase, and on examination of Fig. 8 we find that this is precisely what does happen.

Thirdly, we can predict the complete curve for the whole animal. It is well known that once all the grease has been removed by solvent action from cockroach cuticle, the water permeability of the remaining cuticle is so great that, whatever temperature dependency it may have, it cannot have had any effect on the temperature dependency of the impermeable grease layer, for the cuticle is several orders more permeable at all temperatures. From Fig. 5 we have already noted the considerable temperature dependency of the permeability of thick grease, both below and above transition; in Fig. 8 the 'monolayer' film certainly has temperature dependency above transition, but significantly little below it. It is, therefore, proposed that in the absence of an 'organizing' substrate, such as water or a wet surface, the grease will be in the molecularly unorganized state at temperatures both above *and below* the critical temperature. Once again it is necessary to work in 'resistivity' rather than impermeability units. In Fig. 9*a*, we plot the reciprocal of the absolute permeability of the monolayer below the transition—assuming it to remain constant with temperature—and we plot above the transition the reciprocal of the permeability of a fluid grease layer of equivalent thickness. From the slope of the cuticle temperature curve above transition in Fig. 5 we can obtain an estimate of the temperature dependency of permeability of grease entirely in the fluid state. We assume that such a temperature coefficient of permeability holds also for grease in the molecularly unorganized state *below* the critical temperature. If due account is taken of scales and units, the order of temperature dependency of the 'monolayer' film on water above the transition region is similar. We can then, in Fig. 9*a*, also plot the theoretical curve for the resistance at various temperatures of a layer of grease (without molecular organization) which is twenty-four times as thick as the 'monolayer', basing our values only on the calculated absolute permeability of unorganized grease at 25° C., and the apparent temperature dependence.

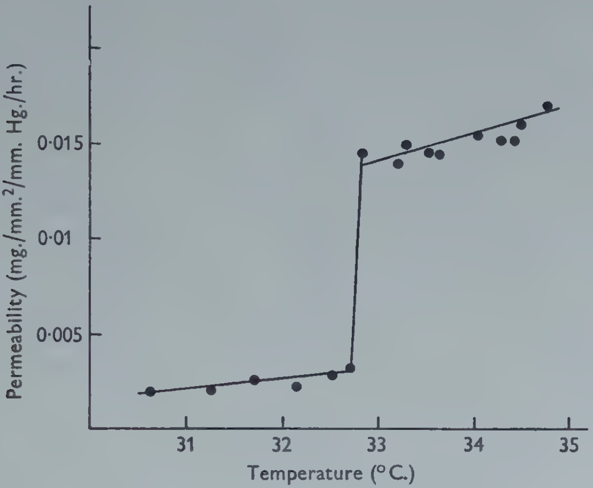

Fig. 8. Graph showing the results of Fig. 7 corrected to permeability per unit saturation deficiency of the air at each experimental determination.

Now these two 'resistance' characteristics can be added giving the uppermost curve in Fig. 9*a*, representing the change of resistance with temperature for the combined layers, i.e. the whole waterproofing system of the natural cuticle. The reciprocal of this curve, plotted in Fig. 9*b*, thus gives the theoretical picture for permeability of the whole cuticle against temperature and can be compared with the 'cuticle' curve of Fig. 5. It will be seen that in *pattern* they are very similar indeed; in detail, the magnitude of the vertical excursion at the transition temperature is appreciably greater in the experimental curve. It must, however, be remembered that one has no idea of the amount of grease on the individual on which the experiments illustrated in Figs. 4 and 5 were carried out; all our calculations above have been made on the assumption that the mean thickness of grease on intact animals is 0·25 μ, but if in fact this individual had less total grease, then this would cause the relative magnitude of the vertical region to be greater, and thus to depart more from the theoretical curve we have derived, though without in any way altering the general pattern of the curve (see Fig. 9*b*).

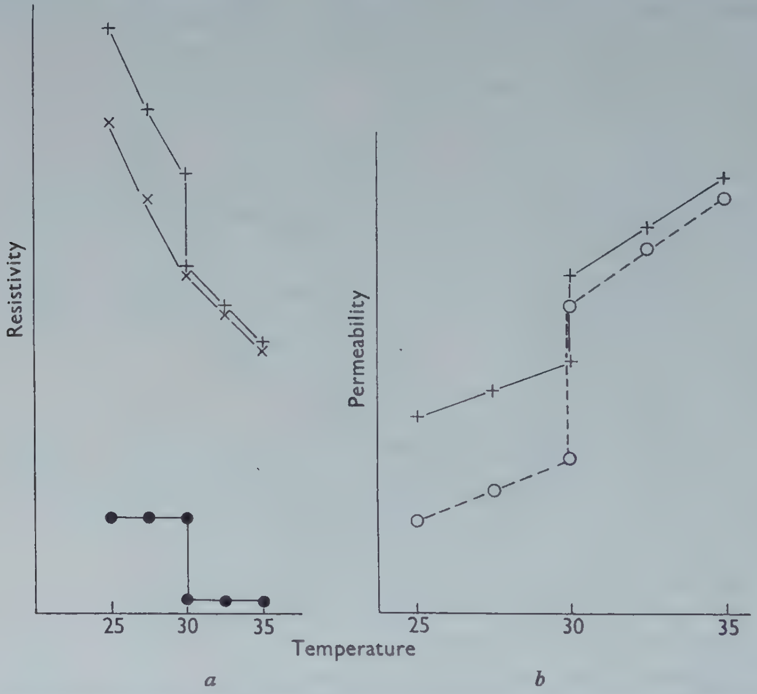

Fig. 9. Theoretical derivation of the form of the transpiration/temperature curve for intact animals. 9*a*. ●, resistivity (thickness divided by absolute permeability) of a 'monolayer' of grease on an aqueous substrate below a transition point at 30° C. and changing to unorganized grease above 30° C. ×, resistivity of a layer of unorganized grease molecules twenty-four times the thickness of the 'monolayer'. +, combined resistivity of a system made up of these two layers, obtained by adding the two previous curves. 9*b*. +, theoretical permeability/temperature curve obtained by taking the reciprocal of the combined resistivity curve of 9*a*. ○, and broken line; the curve of Fig. 5. relating to cuticle temperature converted to the same scale.

These three examples, in which calculations based upon a theoretical picture of the physico-chemical form of the grease and of its changes with temperature have been compared quantitatively with the practical measurements, show—and are only expected to show—agreement to within an order of magnitude. They help to confirm, rather than form the basis for, the proposed explanation. If, as it would appear, the permeability of the 'bulk' grease changes continuously and quite appreciably with temperature, and the fundamental cause of this change is thermal agitation of the molecules of the fluid, then it is similarly likely that the forces causing the grease molecules to be organized against a hydrophilic surface would resist such agitation, and that in consequence the 'monolayer' would have little temperature dependence. What one would like to do is to measure the true permeability of grease entirely in the unorganized state, at a temperature well below the transition, but the way to do this is not obvious. There is of course also the possibility that the portion of the thick grease immediately over the organized layer would itself be to some extent organized, so that the 'monolayer' effect in a thick grease system would be greater than our simplest explanation would suggest.

The existence of an organized layer of grease molecules at the water interface certainly explains some observations made during this study, and which it is proposed to report in detail elsewhere. If the permeability of a grease-covered drop is determined in dry air at a temperature well below transition, and the drop is then immediately exposed to a humidity of, for example, 60% R.H. at the same temperature, its rate of water loss may decrease, or it may increase to two or three times its value in dry air. Fortunately for the experiments reported earlier, the behaviour of all waterproofed droplets is consistent at 0% R.H. The random behaviour of droplets at higher humidities proved difficult to explain, but it was eventually discovered that the recorded rate of evaporation was always proportional to S.D., if the measurements were made in dust-free air. It would appear that dust particles—and especially those of material capable of adsorbing water—can materially interfere with the resistance to water movement through a grease film. The organization of the grease layer we have presumed to depend on the affinity for water of polar endings, such as the hydroxyl groups at the end of long-chain alcohols. A hydrophylic surface above the 'monolayer' could likewise attract these polar endings and compete for them with the water droplet's surface. In dry air particles of dust on the grease film will be dry and so no competition can take place; but at higher humidities, water could be adsorbed on the surface of the dust, and produce a surface attracting some of the polar groups away from the droplet. If this causes such local disorientation, there would be a form of pinhole diffusion through areas of high permeability, so that the magnitude of the observed effect is not altogether surprising.

If then we accept the theory that an organized and orientated 'monolayer' covers the aqueous substrate of the cuticle surface, a physico-chemical issue of some importance arises; because of this, the term 'monolayer' has heretofore been used in parentheses. Whereas in 1945, Beament suggested that the high impermeability of the hard waxes of typical cuticle was due to the existence of a layer of molecularly organized wax, whose increased permeability at transition was caused by a Müller (1932) transformation in the intermolecular spacings, the author now postulates, for the soft grease of the cockroach, something rather more drastic in disorganization; both ideas demand a 'monolayer' type of organization in a substance which is chemically a complex mixture. But it has been suggested on many occasions that mixtures do not in general form organized monolayers in the sense that pure substances do (see, e.g. Adam (1941)). It is quite clear (Beament, 1955) that cockroach grease is a mixture of very special properties and that the natural solvents in it have unique effects; yet the hard waxy components which comprise the other part of the grease are substantially the same as the hard cuticular wax of other insects which have so far been examined. The criticism that complex mixtures do not form 'monolayers' and show changes in crystal form at critical temperatures has not been based only on theoretical or general grounds. Holdgate & Seal (1956) for example, using X-ray and electron-diffraction techniques, failed to find any evidence of sudden changes in samples of wax heated through appropriate temperature ranges; but it has already been pointed out (Beament, 1954), and still is suggested, that organization of this kind is only formed against, and in the presence of, a water substrate which clearly cannot be present in samples studied by these methods. Preliminary tests which the author has made with electrical techniques are, however, promising and free from such objection.

When we now consider the shift in transition point with the age of the cockroach nymph within the instar (p. 506), it is evident that some progressive change affecting the determination of the transition temperature itself is going on. Beament (1955) suggested that by driving off the solvent from the grease one could get samples with successively higher critical temperature up to about 40° C., using a rather crude waterproofed droplet technique. Eventually a hard wax melting between 50 and 55° C. was obtained. It now seems even more likely that as the solvent naturally evaporates from the nymph, during an instar, so the accumulation of higher proportions of the long-chain components in the grease leads to the increased transition temperatures. What now becomes clear is not only that the 'monolayer' is the region of extreme impermeability, but also that its stability and eventual breakdown may be directly governed by the actual proportion of the components in it. In this light Table 4 of Beament (1955) assumes considerably greater importance. Of the insects so far examined the cockroach is unique in that after heating to regions of high permeability and then cooling to ordinary temperatures it alone seems to recover its full impermeability; below the critical temperature there is no reason why a 'monolayer' should not be reformed under the influence of the wet substrate. On the other hand, polar solvents such as chloroform, which could interfere with 'monolayer' formation, certainly do cause permanent damage to waterproofing —damage which can only be made good experimentally by causing the chloroform to evaporate and then using octane-octyl alcohol vapour to replace the natural solvent. The natural solvent, in fact, seems essential for the stabilization of the 'monolayer', and there is (Table 4, Beament 1955) a very strong suggestion that this solvent can cause high impermeability to be restored in cuticles with hard wax (such as those of *Rhodnius*) which have previously been damaged by heat or polar solvent action. But again it must be remembered that none of these things can happen with dead, dry cuticle; it is essential apparently that the surface of the cuticle against the wax, where we may imagine a polarized layer to be formed, should be fully hydrated, once again pointing to the extreme importance of the hydrophilic surface of the cuticle.

CONCLUSION

The waterproofing grease on the cuticle of *Periplaneta americana* would seem to consist mainly of molecularly unorganized lipoids, but (at lower temperatures) to have a layer immediately against the aqueous substrate of the cuticle which is extremely impermeable to water, even though its thickness is of molecular dimensions. At a certain temperature, which undoubtedly depends on the age of the nymph since it last moulted and thus on the relative proportion of volatile and non-volatile components in the grease, this impermeable layer breaks down, causing a sudden increase in the overall water loss of the animal, and the order of change in permeability is consistent with the transition of this organized layer into a state of molecular disorganization similar to that in the main body of grease above it.

The waterproofing of insects having hard cuticular waxes (i.e. the majority of insects), will be investigated as soon as suitable high-temperature techniques have been developed; but it is clear from the form of the curves already published by Wigglesworth, Edney, Holdgate, Beament, etc., that when proper physical measurements are made, the true temperature characteristics of permeability of the hard-waxed cuticles could reveal discontinuities of a similar nature.

SUMMARY

1. Previous methods for determining the temperature characteristics of the permeability to water of insect cuticle are criticized on five fundamental points, and in particular it is shown that they do not measure the temperature of the cuticle which will be substantially different from that of the surrounding air.

2. The physical principles for accurate measurement are outlined, involving the accurate control of air temperature and air circulation, the measurement of cuticle temperature and of water loss within a sealed enclosure, and the assessment of true saturation deficiency. Apparatus for meeting these requirements and making these measurements is described.

3. Determinations have been made of the permeability of the cuticle of *Periplaneta americana* nymphs. When the permeability is plotted against cuticle temperature a sudden and substantial increase in permeability is seen to take place at around 30° C. The temperature at which this change occurs is higher the longer the time which has elapsed since the nymph previously moulted.

4. A method of measuring the permeability of the cuticular grease, when spread as a layer over an isolated water droplet, is described. This layer, whose thickness is of molecular dimensions, has much greater absolute impermeability to water than the thick grease on the insect. It also exhibits a sudden increase in permeability at about 30° C., and the order of change is considerably greater than that found with intact cuticle.

5. The findings are quantitatively compatible with the postulate that, at lower temperatures, the grease on the cockroach consists of an orientated 'monolayer' organized by the attraction of its polar groupings for the aqueous cuticle surface underlying it, together with a much greater thickness of unorganized grease above it. In absolute units, the grease is about five times as permeable to water as is the 'monolayer', but at the transition temperature the measurements agree with the supposition that the molecular organization of the 'monolayer' breaks down into the disorganized state of the major part of the grease with a corresponding increase in permeability.

6. The suggestion that films of grease on water, and the lowermost portion of the grease on the animal, have orientated molecular organization is supported by some observations on the effect of dust particles on the films and by information previously published.

I am especially indebted to Drs R. H. J. Brown and K. E. Machin for their unstinted help with the technical and physical problems involved in this work. I have been fortunate in being able to discuss various aspects with Drs J. Dainty, A. D. Lees and J. A. Ramsay. Finally, I must thank Prof. V. B. Wigglesworth for his long-continued interest and advice.

REFERENCES

ADAM, N. K., (1941). *The Physics and Chemistry of Surfaces.* Oxford University Press.

BEAMENT, J. W. L. (1944). An apparatus to measure contact angles. *Trans. Faraday. Soc.* **41**, 45.

BEAMENT, J. W. L. (1945). The cuticular lipoids of insects. *J. exp. Biol.* **21**, 115.

BEAMENT, J. W. L. (1946). The waterproofing process in eggs of Rhodnius proxilus Stahl. *Proc. Roy. Soc.* B, **133**, 407.

BEAMENT, J. W. L. (1951). The structure and formation of the egg of the fruit tree red spider mite, *Metatretanychus ulmi* Koch. *Ann. appl. Biol.* **38**, 1.

BEAMENT, J. W. L. (1954). Water transport in insects. *Symp. Soc. exp. Biol.* **8**, 94.

BEAMENT, J. W. L. (1955). Wax secretion in the cockroach. *J. exp. Biol.* **32**, 514.

BEAMENT, J. W. L. (1958*a*). The measurement and control of temperature. *Electronics for Biologists*, chap. 29. Butterworth. (In the Press).

BEAMENT, J. W. L. (1958*b*). The measurement and control of humidity. *Electronics for Biologists*, chap. 30. Butterworth. (In the Press).

BEAMENT, J. W. L. & MACHIN, K. E. (1958*a*). A new method for the regulation of air temperatures. *J. sci. Inst.* (in the Press).

BEAMENT, J. W. L. & MACHIN, K. E. (1958*b*). British Prov. Pat. Spec. No. 5184/58.

BARRER, R. M. (1941). *Diffusion in and through Solids.* Cambridge.

EDNEY, E. B. (1951). The evaporation of water from woodlice and the millipede *Glomeris*. *J. exp. Biol.* **28**, 91.

HOLDGATE, M. W. (1955). The wetting of insect cuticles by water. *J. exp. Biol.* **32**, 591.

HOLDGATE, M. W. (1956). Transpiration through the cuticles of some aquatic insects. *J. exp. Biol.* **33**, 107.

HOLDGATE, M. W. & SEAL, M. (1956). The epicuticular wax layers of the pupa of *Tenebrio molitor* L. *J. exp. Biol.* **33**, 82.

JEFFREYS, H. (1918). Some problems of evaporation. *Phil. Mag.* **35**, 270.

MÜLLER, A. (1932). An X-ray investigation of normal paraffins near their melting-points. *Proc. Roy. Soc.* A, **138**, 514.

PASQUILL, F., (1949). Eddy diffusion of water vapour and heat near the ground. *Proc. Roy. Soc.* A, **198**, 116.

RAMSAY, J. A. (1935). The evaporation of water from the cockroach. *J. exp. Biol.* **12**, 373.

RAYLEIGH, LORD (1896). On the stability or instability of certain fluid motions. *Collected papers* **4**, 216.

TAYLOR, R. L., HERRMANN, D. B. & KEMP, A. R. (1936). Diffusion of water through insulating materials. *Industr. Engng Chem.* **28**, 1255.

WIGGLESWORTH, V. B. (1945). Transpiration through the cuticle of insects. *J. exp. Biol.* **21**, 97.

WIGGLESWORTH, V. B. (1947). The epicuticle of an insect, *Rhodnius prolixus*. *Proc. Roy. Soc.* B, **134**, 163.

WIGGLESWORTH, V. B. (1948). The structure and deposition of the cuticle in the adult mealworm *Tenebrio molitor* (Coleoptera). *Quart. J. micr. Sci.* **89**, 197.

THE INDUCTION AND TERMINATION OF FACULTATIVE DIAPAUSE IN THE CHALCID WASPS *MORMONIELLA VITRIPENNIS* (WALKER) AND *TRITNEPTIS KLUGII* (RATZEBURG)*

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Many insects exhibit a facultative diapause which is under the control of specific environmental factors. When the insect enters diapause growth and development cease. The end of diapause is signalled by the resumption of development. In most instances the environmental agencies that cause diapause affect the generation that will enter diapause, but occasionally a stimulus applied to the maternal generation affects the post-embryonic stages of the progeny. Commonly in facultative diapause, normal growth proceeds for several moults after the application of the stimulus, and development ceases only at a later stage. Whatever the mechanism which finally prevents moulting and produces diapause, it does not interfere with intermediate moults. The present report defines the extrinsic factors that cause facultative diapause in the last larval instar of two parasitic wasps and analyses in detail the mechanism of diapause termination.

MATERIALS AND METHOD

(1) *Experimental animals*

Mormoniella vitripennis (Walker) (=*Nasonia brevicornis* Ashmead) and *Tritneptis klugii* (Ratzeburg) (=*Caelopisthia nematicida*) are closely related chalcid wasps in the family Pteromalidae. Both have a facultative larval diapause (Girault & Saunders, 1909; Graham-Smith, 1919; Altson, 1920; Cousin, 1932; Evans, 1933; Roberts, 1935; Van der Merwe, 1945; Moursi, 1946*b* (*Mormoniella*); Hewitt, 1912 (*Tritneptis*)). *Mormoniella* has been the subject of considerable genetic (e.g. Whiting, 1949, 1955*a–d*) and some physiological study (e.g. Jacobi, 1939; Moursi, 1946*a*, *b*; Schneiderman & Williams, 1952; Schneiderman, Horwitz & Kurland, 1956; Schneiderman, Kuten & Horwitz, 1956; Ray, Hunter & Stephens, 1954; Ferschl & Wolsky, 1956). Its post-embryonic development has been described in great detail by Tiegs (1922) and its general biology by various workers (Girault & Saunders, 1910; Roubaud, 1917; Parker, 1924; Parker & Thompson, 1928; Cousin, 1933; Fukuda, 1939). *Tritneptis* is currently used to control saw-fly damage to

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spruce trees in Northern Ontario, but little is known of its biology (Hewitt, 1912). Both wasps are external parasites upon other insects, are easily reared in the laboratory, and at room temperature have a life cycle of about 2 weeks. They are relatively flightless and can be handled with a fine camel-hair brush. *Mormoniella* can be reared at temperatures from 15 to 30° C. whereas *Tritneptis* thrives only below 30° C.

The life history of *Mormoniella* is typical of both wasps and is briefly described below. The female is parasitic on pupae of muscoid Diptera like the flesh-fly *Sarcophaga bullata* (Parker). After piercing the puparium she lays her eggs on the developing fly pupa. One to three days later the egg hatches and the first-instar larva begins feeding as an ectoparasite on the fly pupa. The larva feeds and moults four times (Van der Merwe, 1945) during the next 3 days, finally ceases feeding and enters a resting stage which persists for about 24 hr. The larval tissues then begin to break down and simultaneously the imaginal disks proliferate. Only a few cell divisions are necessary to break down the thin partition between the mid-gut and the invaginated rectum, and defaecation next occurs. The pre-defaecating larva is greyish in colour but becomes white when faecal material is voided. Defaecation marks the end of the resting stage. The short post-defaecation period (20 hr.) is occupied by the great cellular activity of prepupal development and terminates with the pupal moult. Within 12 hr. the newly moulted white pupa becomes pinkish in colour. Epidermal differentiation and pigmentation then proceed for about 5 days, whereupon the fully formed adult wasp resorbs its moulting fluid and emerges. The time sequence for the development of *Mormoniella* at 25° C. from egg to adult is presented in Table 1, and typical developmental stages are illustrated in Fig. 1. At room temperature the adult will live without food for about 6 days. Females supplied with fly puparia survive about twice as long, feeding on the pupal fluids. Eggs are laid from the second day after adult emergence until death.

Table 1. *Time-table for the development of* Mormoniella *at* 25° C.

Day	Stage
0	Egg
2–3	First-instar larva
5–6	Last-instar larva
6–7	Larval defaecation
8	White pupa
9	Pink pupa
10	Red eyes
11	Red-brown eyes
12	Black head and thorax
13	All black
14	Adult emergence

The life history and development of *Tritneptis*, which is summarized in Table 2, follows a similar pattern except that the hosts are saw-fly prepupae.

When diapause occurs in either species it is manifest at the end of the last larval instar, after the feeding period has ended and just prior to defaecation. The diapausing larva differs from the normal larva in appearance. The fat-body becomes semi-transparent (Van der Merwe, 1945) and, as first pointed out by Whiting (personal communication), the cuticle acquires a sticky waxy coating. But the truly diagnostic feature of the diapausing larva is that it does not immediately moult into a pupa. These diapausing larvae will survive for many months at room temperature. In the case of *Mormoniella*, diapausing larvae kept at room temperature for

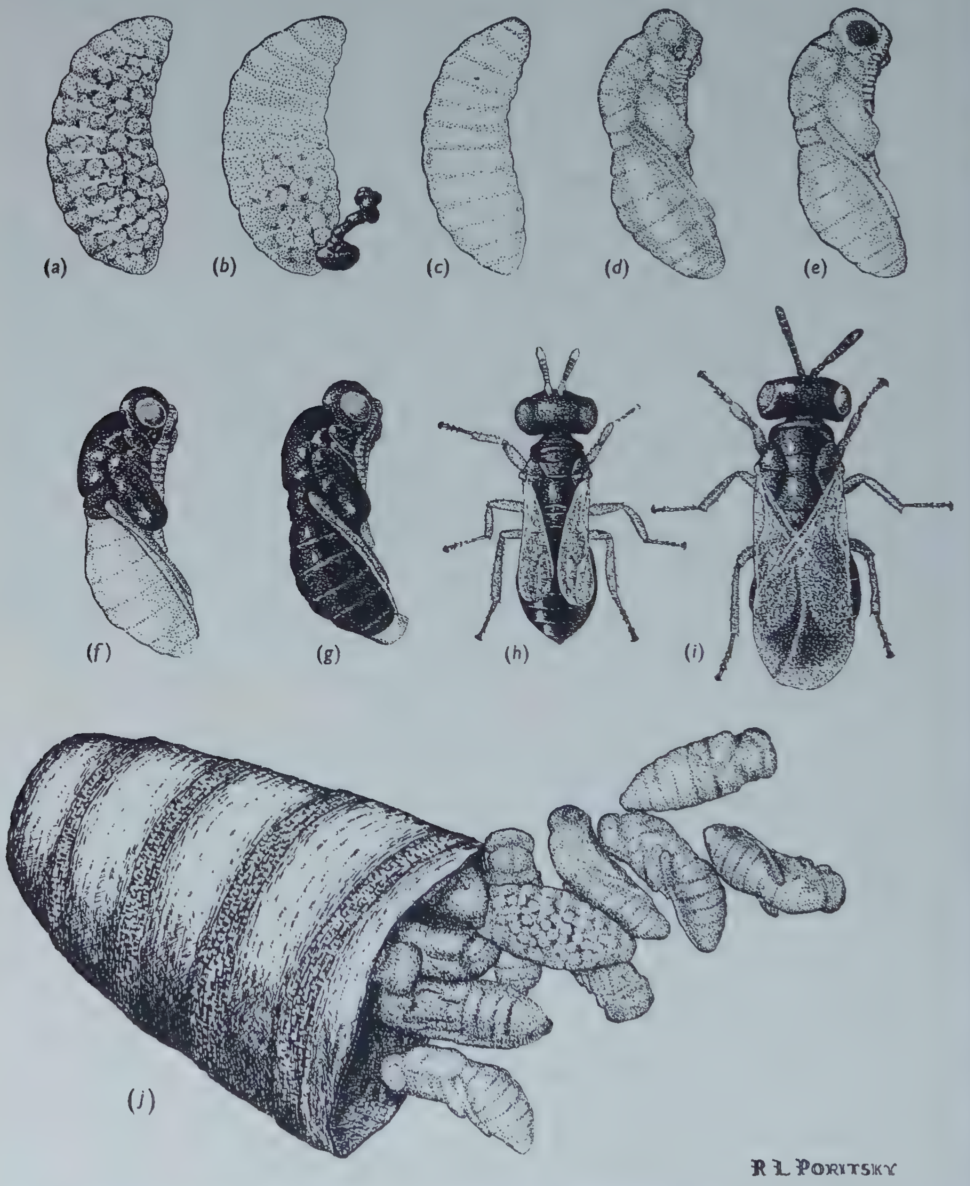

Fig. 1. Stages in the development of *Mormoniella*. (*a*) Diapausing larva. (*b*) Defaecating larva. (*c*) Early prepupa. (*d*) Pink pupa. (*e*) Red eyes. (*f*) Black head and thorax. (*g*) All black. (*h*) Adult male. (*i*) Adult female. (*j*) *Sarcophaga* puparium broken open to reveal enclosed diapausing larvae and pupae. The size of the larva is 2·2 mm.

more than 2 years have been capable of developing into normal adult wasps when appropriately stimulated. Either larval defaecation or the pupal moult may serve as convenient end-points for diapause, although other changes such as wrinkling of the larval cuticle are evident even earlier.

Table 2. *Time-table for the development of* Tritneptis *at* 25° C.

Day	Stage
0	Egg
1–2	First-instar larva
8–9	Larval defaecation
10	Orange pupa
11	Red eyes
12	Brown eyes
13	Black head and thorax
14	Striped abdomen
15	All black
16	Adult emergence

Except where specified, an inbred strain of *Mormoniella*, 'Woods Hole wild-type',* and a wild strain of *Tritneptis*† were used. In a few experiments a strain of *Mormoniella* collected locally, 'Ithaca wild-type', and several mutant strains were employed.

(2) *Hosts*

The flesh-fly *Sarcophaga bullata* (Parker) served as host for *Mormoniella*. Flies were raised on raw hamburger or brewers' yeast and powdered milk, permitted to develop at room temperature for 4–5 days after pupation and then stored at 6° C. Animals treated in this manner can emerge after several months of storage and the puparia can be used for host material for a year.

Tritneptis, a parasite of saw-flies, was raised upon the larch saw-fly, *Neodiprion lecontei*.† *Lecontei* saw-flies also enter diapause, and the diapausing prepupa encased in a tough cocoon normally served as host material. These cocoons may be stored at temperatures as high as 6° C., but pupation occurs after several months and storage at a temperature of 2° C. is preferable. An attempt was made to raise *Tritneptis* on *Sarcophaga*. The wasps managed to parasitize the fly puparia, and while occasionally an individual completed its development it was stunted and failed to produce another generation on *Sarcophaga* (cf. Cousin, 1933, for *Mormoniella* on *Galleria*).

(3) *Rearing conditions*

Wasps were reared in cotton-plugged vials. All vials were kept in opaque cardboard containers, unless specifically stated otherwise, and stored in incubators regulated to within 1° C. and maintained at 70% humidity. In a few experiments where more precise thermo-regulation was required a water-bath was employed and the vials were placed in large cotton-plugged opaque flasks.

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(4) *Procedure for anaerobic experiments*

In several experiments both adults and diapausing animals were kept without oxygen for specific periods. Quarter-litre flasks furnished with a side-arm and two stop-cocks were used. Animals were enclosed in the flask to which was added 25 ml. of a powerful oxygen absorbent (Fieser, 1924). The flasks were then promptly flushed with nitrogen (99·9%) and sealed. In all but a few experiments a small vial containing a saturated solution of lead acetate and a fluted filter paper were added to absorb the small amounts of hydrogen sulphide liberated by the Fieser solution.

THE INDUCTION OF DIAPAUSE IN *MORMONIELLA*

Both in nature (Altson, 1920) and in mass laboratory cultures at 25° C. a persistent but irregular proportion of *Mormoniella* larvae enter diapause. In individual matings at 25° C. a similar irregular incidence of diapause is commonly found. A single female may produce both diapausing and non-diapausing offspring in the same puparium, or sometimes only one or the other. During the past 6 years experiments were conducted with the offspring of more than 5000 individual females to uncover factors that increase or decrease the incidence of this facultative diapause. Studies were made of the effects on the offspring of nutrition, temperature, humidity, photoperiod, etc. These extrinsic factors, as well as the intrinsic ones of heredity, age, size, etc., were also appraised in the maternal generation, in order to determine the presence of a maternal influence. This report summarizes the principal findings.

(1) *Factors affecting the larval generation*

(A) *Superparasitization*

It had been suggested by Cousin (1932) that successive parasitization of the same host puparium caused developing larvae of *Mormoniella* to enter diapause because of the reduced food supply available to them. This seemed unlikely because of the frequent occurrence of puparia containing only two or three plump diapausing larvae and no other individuals, i.e. diapause amidst ample food. Nevertheless, the possibility was tested by parasitizing individual puparia on 3 successive days with wild-type *Mormoniella* and two eye-colour mutants, oyster-eye and orange-eye (*oy* and *or*—336·2 of Whiting, 1955*b*). The experiment was conducted at 30° C. No diapausing larvae were produced, although many puparia had been superparasitized and yielded all three kinds of adults.

(B) *Age and condition of host*

In some hymenopterous parasites diapause is thought to depend upon the stage of development of the host at the time of parasitization (Flanders, 1944). This is not the case in *Mormoniella*, for the incidence of diapause was not affected by the age of the developing fly within the puparium, nor by the length of time the puparium had been stored in the cold. Duplicate experiments were conducted at 25 and

30° C., and the same proportion of diapause occurred in fresh puparia of various ages and in puparia which had been stored at 6° C. for almost a year.

In an effort to decrease the nutritional value of the host, puparia were damaged by puncturing the head region and the injuries sealed with paraffin to reduce desiccation. Although the punctured pupae soon died they could commonly be parasitized for at least 1 day after damage. Few wasps developed within each punctured puparium because of rapid desiccation and decay, but those surviving on this obviously poor host material showed no increased tendency to enter diapause.

(C) *Humidity and photoperiod*

Parasitized puparia were placed at relative humidities varying from about zero ($CaSO_4$) to nearly 100% (water) at 25 and 30° C. The incidence of diapause was the same at all humidities tested. The exposure of parasitized puparia to varying photoperiods also failed to affect the frequency of diapause.

(D) *Temperature*

Puparia at various times after parasitization at 25 and 30° C. were kept at 15, 20, 25 or 30° C. The rates of development naturally varied with the temperatures, but the incidence of diapause was the same at all temperatures. Thus the temperature at which larval development took place did not affect the incidence of the diapause.

In further experiments several extrinsic factors were varied simultaneously, e.g. temperature, humidity, etc., without effect. Since the extrinsic conditions to which larvae were exposed failed to affect the incidence of diapause, attention focused on the maternal generation.

(2) *Factors affecting the maternal generation*

(A) *Intrinsic factors*

Careful analysis of the frequency of diapause in the offspring of females during their entire life at several temperatures revealed no correlation between the age of the female and the incidence of diapause. Fecundity was similarly studied and there was no difference in the frequency of diapause offspring between highly fecund and almost infertile females.

The significance of size was next examined to determine whether larger females produced a different proportion of diapausing offspring than smaller females. Animals were divided into two groups: those measuring 2·3–2·6 mm. in length and those measuring 1·8–2·0 mm. The proportion of diapause in the offspring of these two groups was not significantly different. Finally, an effort was made to select out a diapause-producing strain, but this too proved fruitless. Diapause was evidently phenotypic (but see Discussion for experiment of Moursi (1946*b*)). Our attention then turned to the effects of extrinsic agencies on the maternal generation.

(B) *Host deprivation*

In addition to ovipositing on *Sarcophaga*, female *Mormoniella* commonly feed on the pupal juices through punctures made with their ovipositors. Depriving females of hosts thus results in both starvation and possibly also oösorption (Flanders, 1944) and reduces by half their normal life span of about 2 weeks at 25–30° C. In order to ascertain the effects of host deprivation on the incidence of diapausing progeny, the following experiment was carried out at 30° C. Into each of twenty small vials were placed a newly emerged male and female *Mormoniella*. Two *Sarcophaga* pupae were added to each of five vials after 1, 2, 3 or 5 days, and daily thereafter until the twelfth day.

Table 3. *The effect of various periods of host deprivation on the incidence of diapause in the progeny of* Mormoniella *females maintained at* 30° C.

Days of deprivation	Total offspring	% diapause
1	1786	0
2	2125	34·6
3	1992	67·1
5	1866	97·3

The data summarized in Table 3 reveal a correlation between host deprivation and the incidence of diapause. The first group showed no diapause. In the group that was deprived for 2 days one animal produced only diapause offspring, three produced only developing animals; one showed an intermediate condition producing both developing and diapausing offspring on the first day and thereafter only diapausing ones. Females deprived of hosts for 3 days produced a still greater proportion of diapause offspring, and females deprived for 5 days produced nearly 100 % diapausing progeny for the duration of the experiment.

In this experiment 5 days without food caused a profound change in the physiology of the female as measured by the frequency of diapause in her offspring. In subsequent experiments the results were not so clear-cut, but there was, nevertheless, a strong tendency for starved females to produce more diapausing offspring.

(C) *Chilling the female*

Newly emerged males and females from a continuously breeding population were placed in pairs in small vials; five pairs were stored at 25° C. throughout and five pairs were exposed to 10° C. for 5 days and then stored at 25° C. Puparia were present throughout the experiment but no oviposition or feeding took place at 10° C. Puparia were collected for about a week at 25° C. In this experiment, in the group exposed to 10° C. prior to oviposition, all of the progeny produced at 25° C. entered diapause, while in a group kept continuously at 25° C., 30% of the animals developed without arrest. A second experiment was performed in identical fashion with newly emerged animals which had been in diapause for more than 100 weeks and then permitted to develop at 25° C. The wasps reared at 25° C. throughout yielded no diapause offspring, while the wasps chilled for 5 days and then returned to 25° C. produced 21% diapause offspring. These two experiments suggest that chilling a female enhances the chance that her progeny will enter diapause. To be sure, there are large differences in the proportion of diapause between the experiments, but in these experiments and in numerous others like them, chilling of the females invariably led to a marked and significant increase in the incidence of diapause in their offspring.

More detailed experiments were conducted to analyse this chilling effect and to determine what developmental stage of the female was sensitive to low temperature. To this end, animals at the following developmental stages (see Table 1) were placed at 10° C. for 5 days: red-brown eye pupae, black head and thorax pupae, black pupae, newly emerged adults, 1-day-old adults, 2-day-old adults. Each group consisted of six vials with one mating pair per vial. After the 5-day period of chilling five vials from each group were placed at 30° C. and the sixth at 25° C. Two *Sarcophaga* puparia were added to each vial daily. The results recorded in Table 4 reveal that chilling had no effect upon red-brown eye pupae or upon pupae at the stage of black head and thorax: all of their offspring developed into adults without the intervention of a diapause. The group of all black pupae also produced only developing progeny, with the exception of one female, which was observed to have emerged as an adult before the group was removed from the low temperature. This animal first gave rise to both diapausing and developing progeny and then produced diapausing progeny exclusively. This foreshadowed the results in the next group, newly emerged adults, where all of the progeny produced immediately after chilling entered diapause. In three of the four fertile animals, the chilling effect began to wear off after several days: that is, they produced developing animals after a period of producing only diapausing ones. Similar results were obtained with the 1-day-old and 2-day-old adults. Table 5 records the fate of the animals placed at 25° C. instead of 30° C. after chilling. The results are substantially the same as those of Table 4, although confused somewhat by a high spontaneous incidence of diapause at 25° C., which will be considered presently.

The temporary nature of the chilling effect was clearly seen in numerous experiments in which females were permitted to oviposit at 30° C. for several days, exposed to 10° C. for 5 days and then returned to 30° C. for further oviposition. Such females produced no diapausing offspring, or only a few, before chilling. After chilling they produced many diapausing offspring for several days and then returned to producing developing offspring. Control experiments showed that low temperature and not simply change of temperature was important.

In addition to the clear effects of chilling adults in inducing diapause, comparison of the 25 and 30° C. groups (Tables 4 and 5) showed that oviposition and rearing at 30° C. suppressed the normal 'spontaneous' incidence of diapause at 25° C. This fact was confirmed in the following experiments.

Thirty mating pairs, which had developed at 25° C. until emergence, were stored individually at 15° C. where they were permitted to oviposit daily. Each day three groups of ten parasitized puparia were taken; one group was stored at 15° C., another group was stored at 25° C. and another at 30° C. The data recorded in Table 6 reveal the same results at all three temperatures: oviposition at 15° C. caused almost all the progeny to enter diapause. The immediate removal of the eggs

Table 4. *The effect of chilling thirty* Mormoniella *females at various stages of adult development for 5 days at* 10° C. *on the incidence of diapause in progeny oviposited and reared at* 30° C.

Days	Number and kind of progeny*									
	Red-brown eye stage					Black head and thorax stage				
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	34*a*	0	42*a*	35*a*	0
3	35*a*	0	40*a*	0	26*a*	37*a*	0	60*a*	59*a*	0
4	28*a*	63*a*	45*a*	0	37*a*	31*a*	49*a*	62*a*	72*a*	38*a*
5	36*a*	41*a*	36*a*	0	42*a*	57*a*	61*a*	40*a*	64*a*	21*a*
6	39*a*	55*a*	24*a*	0	49*a*	71*a*	36*a*	52*a*	57*a*	48*a*
7	44*a*	56*a*	37*a*	0	58*a*	54*a*	20*a*	58*a*	56*a*	0
8	34*a*	0	7*a*	0	0	39*a*	30*a*	47*a*	49*a*	0
9	45*a*	0	11*a*	0	0	46*a*	58*a*	55*a*	40*a*	0
	Total offspring, 888; % diapause, 0					Total offspring, 1578; % diapause, 0				
Days	All black stage					Newly emerged adults				
1	0	0	0	0	0	0	0	0	0	0
2	30*a*	0	41*d*; 11*a*	0	0	0	0	0	57*d*	0
3	71*a*	0	44*d*	57*a*	0	49*d*	64*d*	52*d*	58*d*	0
4	32*a*	0	63*d*	83*a*	0	43*d*	67*d*	51*d*	60*d*	0
5	23*a*	30*a*	40*d*	53*a*	41*a*	44*d*	53*d*	49*d*	46*d*	0
6	58*a*	55*a*	49*d*	52*a*	50*a*	0	66*d*	54*d*	40*d*	0
7	0	56*a*	41*d*	0	53*a*	50*d*	49*d*	56*d*	11*d*; 10*a*	0
8	53*a*	49*a*	53*d*	47*a*	42*a*	59*d*	50*d*; 12*a*	19*a*	30*a*	0
9	52*a*	42*a*	0	0	40*a*	49*d*	32*d*; 20*a*	6*a*	32*a*	0
	Total offspring, 1411; % diapause, 23·5					Total offspring, 1338; % diapause, 90·4				
Days	1-day-old adults					2-day-old adults				
1	0	27*d*	21*d*; 30*a*	32*d*	40*d*	0	47*d*	0	32*d*	43*d*
2	30*d*	40*d*	52*a*	47*d*	47*d*	45*d*	50*d*	0	17*d*	72*d*
3	49*d*	60*d*	42*a*	52*d*	43*d*	38*d*	57*d*	0	54*d*	67*d*
4	28*d*	23*d*	59*a*	31*d*	37*d*	31*d*	59*d*	0	64*d*	59*d*
5	33*d*	47*d*	55*a*	32*d*	52*d*	66*d*	16*a*; 43*d*	0	65*d*	57*d*
6	0	50*d*	62*a*	65*d*	38*d*	52*d*	60*a*	0	24*d*	55*d*
7	40*d*; 3*a*	48*d*	65*a*	51*d*	33*d*	23*d*	51*a*	0	25*d*	48*d*
8	32*d*; 8*a*	37*d*; 18*a*	61*a*	54*d*	29*d*; 5*a*	27*d*	42*d*; 10*a*	0	47*d*	53*d*
9	31*d*; 20*a*	42*d*; 10*a*	0	42*d*	25*d*; 7*a*	22*d*	32*d*; 11*a*	0	52*d*	50*d*; 20*a*
	Total offspring, 1885; % diapause, 72·5					Total offspring, 1686; % diapause, 90·0				

* *a*, non-diapausing progeny that developed without arrest; *d*, diapausing progeny.

for development at higher temperatures failed to influence the subsequent development of these eggs. Once destined for diapause, even 30° C. failed to override the effect of chilling. Chilling may cause only a temporary change in the maternal physiology, but the resulting effect on the egg is apparently an irreversible one. Parallel experiments showed that females that were raised at 25° C. and that oviposited at 30° C. produced eggs that gave rise almost exclusively to developing offspring, regardless of the temperature at which the eggs were incubated. Clearly, low temperature acts on the female and not on the developing offspring.

Table 5. *The effect of chilling six* Mormoniella *females at various stages of adult development for* 5 *days at* 10° C. *on the incidence of diapause in progeny oviposited and reared at* 25° C.

Number and kind of progeny*						
Days	Red-brown eye stage	Black head and thorax stage	All black stage	Newly emerged adult	1-day-old adult	2-day-old adult
1	0	0	0	0	46*d*	41*d*
2	22*a*	47*a*	14*d*; 52*a*	25*d*	35*d*	26*d*
3	52*a*	59*a*	10*d*; 12*a*	0	58*d*	42*d*
4	41*a*	57*a*	40*d*; 21*a*	41*d*	15*d*	30*d*
5	35*a*	16*d*; 42*a*	16*d*; 43*a*	55*d*	50*d*	44*d*
6	65*a*	19*d*; 41*a*	0	29*d*	10*d*	0
7	0	25*d*; 40*a*	0	12*d*	0	48*d*
8	25*d*; 62*a*	27*d*; 54*a*	0	50*d*	0	0
9	15*d*; 56*a*	14*d*; 37*a*	0	0	30*d*; 37*a*	0
10	5*d*; 66*a*	12*d*; 38*a*	0	0	0	0
Total off-spring	444	528	208	212	281	231
% diapause	10·1	21·4	38·5	100	86·8	100

* *a*, non-diapausing progeny that developed without arrest; *d*, diapausing progeny.

Table 6. *The effect of exposing females to* 15° C. *during oviposition on the incidence of diapause in progeny reared at various temperatures*

	Temperature at which progeny were reared		
	15° C.	25° C.	30° C.
Total offspring	4140	2875	2276
% diapause	88·2	100	99·3

These several experiments with temperature have been repeated many times with varying techniques, but with substantially the same results: (*a*) exposing adult females to low temperatures greatly enhances the chance that their progeny will enter diapause; (*b*) the incidence of diapause is greater in the progeny of females ovipositing at lower temperatures; (*c*) the effect of low temperature on the female wears off after a time. The common factor in these experiments appears to be the temperature at which oögenesis takes place; when oögenesis occurs at low temperature, diapause is more likely in the offspring.

(D) *Humidity, photoperiod, social factors*

Although low temperature was an exceedingly important factor in inducing diapause, it might not be the only factor, as was revealed by a careful study of offspring of several hundred females at low temperatures. Occasionally, even in cultures reared at 30° C., an outbreak of spontaneous diapause occurred. Likewise, in cultures reared at 15° C. large numbers of developing adults were sporadically produced. These exceptions led us to suspect that factors other than temperature and nutrition might be involved. Without describing numerous fruitless experiments, suffice it to say that many environmental factors were studied including humidity, photoperiod, as well as social factors, such as population density, numbers of puparia, crowding, etc. No additional combination of factors that increased or decreased the incidence of diapause was disclosed. On the basis of our present data it seems safe to conclude that the temperature history of the adult female is clearly the most critical factor in the induction of facultative diapause in *Mormoniella*, but that other factors probably operate.

THE INDUCTION OF DIAPAUSE IN *TRITNEPTIS*

(1) *Effects of temperature*

The discovery of a successful means of inducing diapause in *Mormoniella* led us to similar experiments with *Tritneptis*. Adults which had emerged at 25° C. were placed at 15, 20 and 25° C. with several *lecontei* cocoons upon which they oviposited. After the parasites had completed their development the cocoons were opened. All of the offspring in the 25° C. group developed promptly into adults, while 99% of the offspring in the 15° C. group entered diapause. The 20° C. group yielded both kinds of animals. The results of this experiment were clear enough except that they did not discriminate between the influence of low temperature on the parent and on the developing larva. Either or both might have responded to the stimulus of low temperature. The following experiment resolved this question. Promptly upon emergence, mating pairs reared at 25° C. were placed in vials at 15 and 25° C. and given cocoons periodically. Immediately after parasitization, cocoons from each group were placed at both 15 and 25° C., where the parasites completed their development. None of the parasites which were placed at 25° C. to complete their development entered diapause, while nearly all of the animals from cocoons which were placed at 15° C. entered diapause. The temperature at which the parents had been raised was of no significance. Low temperature did not act on the parents, but on their offspring: when the larvae developed at low temperature they usually entered diapause; when they developed at high temperatures they never did. Identical results were obtained with adults which had been reared at 15° C. The next experiment was designed to determine how long an exposure to low temperature was necessary and what stages of egg or larval development were sensitive.

Isolated mating pairs which had been reared at 25° C. were given a fresh cocoon daily and kept at 25° C. At intervals of 1, 3 or 5 days after oviposition these cocoons were placed at 15° C. They remained at 15° C. for 5, 15 or 35 days, whereupon they were returned to 25° C. to complete their development. Examination of the cocoons yielded the results summarized in Table 7. Exposure to 15° C. for 5 days was just sufficient to induce diapause in some of the larvae which had remained at 25° C. for 5 days prior to chilling. Most of the larvae that were returned to 25° C. after 15 and 35 days of chilling exhibited diapause. What development there was in these animals invariably occurred in cocoons containing *lecontei* that had pupated prior to parasitization, an observation to which we shall return presently. In this experiment all the *Tritneptis* within a single cocoon behaved in the same manner as far as diapause was concerned.

Table 7. *The effect on the incidence of diapause of exposing* Tritneptis *larvae of various ages to 15° C. for 5, 15 and 35 days*

Days at 25° C. prior to chilling	Days at 15° C. prior to return to 25° C.	Type of larva resulting from treatment	
		Developing	Diapausing
0	0*	+	0
1	5	+	0
3	5	+	0
5	5	+†	+
1	15	0	+
3	15	+†	+
5	15	0	+
1	35	0	+
3	35	+†	+
5	35	+†	+

* Unchilled controls.

† Development occurred only in cocoons that contained *lecontei* which had pupated.

In a parallel experiment, *Tritneptis* that had been reared at 15° C. were permitted to oviposit at this temperature. The parasitized cocoons were then transferred to 25° C. at various times after oviposition. Parasitized cocoons that had been kept at 15° C. for 12 days or less before being placed at 25° C. gave rise to developing offspring exclusively. Cocoons stored at 15° C. for longer than 12 days yielded diapausing animals only. Thus, about 5 days of development at 25° C. and 12 days at 15° C. brought *Tritneptis* larvae to a temperature-sensitive developmental stage, where a period of chilling caused them to enter diapause. The upper limit of this temperature-sensitive period was determined in the following experiment.

Cocoons containing eggs of an approximately known age were obtained by collecting parasitized *lecontei* twice daily from vials containing large numbers of *Tritneptis*. After parasitization the cocoons were kept at 25° C. for from 1 to 8 days and were then stored at 15° C. for 30 days. (*Tritneptis* larvae commonly defaecate on the ninth day at 25° C. (Table 2).) The results summarized in Table 8 reveal that cocoons kept for 8 days at 25° C. before chilling yielded developing offspring only; cocoons stored for only 4 days before chilling yielded diapausing offspring only; at intermediate stages both kinds of offspring were found. Thus chilling animals which have progressed beyond the seventh day of development at 25° C. fails to induce diapause.

Table 8. *The effect on the incidence of diapause of exposing* Tritneptis *larvae of various ages to 15° C. for 30 days*

Days at 25° C. prior to chilling	% of larvae going into diapause
1	100
2	100
3	100
4	100
5	62
6	50
7	20
8	0

Efforts to delimit further the temperature-sensitive period failed principally because of the impossibility of obtaining *Tritneptis* larvae of exactly known age. For although all animals within a single *lecontei* cocoon were commonly at the same stage of development, cocoons exposed to identical conditions frequently contained *Tritneptis* which differed in developmental stage by as much as 2 days. It was therefore virtually impossible to estimate closer than to within 2 days the developmental stage of the larvae within an unopened cocoon. Experiments that required larvae of precise ages were consequently ruled out.

This finding of varying rates of development among wasps in different cocoons probably accounts for the fact that *Tritneptis* from cocoons parasitized at the same time often reacted differently to exposure to low temperature in diapause-induction experiments. These occasional aberrant results are understandable when one recognizes that the larvae were probably in different developmental states.

Recently it proved possible to raise *Tritneptis* on *lecontei* prepupae that had been removed from their cocoons immediately after parasitization and placed in moist chambers. The wasp larvae developed normally, though slightly more rapidly than in the cocoon, and development was not at the same rate in all the parasites of a single prepupa as it was in the enclosed cocoon. This technique should permit selection of larvae of known age and thereby precisely define the temperature-sensitive period.

(2) *Other factors*

Although the experiments just considered showed that chilling of the larva was apparently a sufficient stimulus for diapause induction in *Tritneptis*, other factors were tested to see whether special conditions need accompany chilling and whether another agency could substitute for chilling. Careful study of numerous adult females revealed that size, photoperiod, temperature history, etc., had no influence on the incidence of diapause in their offspring. Similar experiments conducted on larvae failed to reveal factors that could affect or substitute for the action of chilling. The influence of host condition was also assessed by using both *lecontei* prepupae and pupae in various conditions. The only fact that emerged has already been noted, namely, that in some cases chilled *Tritneptis* larvae grown on *lecontei* pupae failed to enter diapause.

These findings lead to the conclusion that chilling *Tritneptis* larvae at a particular stage in larval development induces diapause.

TERMINATION OF DIAPAUSE IN *MORMONIELLA*

At temperatures above 15° C. *Mormoniella* larvae remain in permanent diapause until death ensues after a year or more. However, if the larvae are exposed to temperatures below 15° C. for several months and then returned to higher temperatures, diapause ends and the larval-pupal and pupal-adult transformations take place. In our experience, chilling is the only treatment that will terminate diapause. Exposure to low temperature effects changes within the insect which render it competent to develop. For our present purposes it is not necessary to specify the site of action of low temperature, although it is very likely the brain itself (Schneiderman, 1957 and Discussion). Low temperature permits physiological changes to take place within the insect which prepare the larva for the resumption of morphogenesis when it is returned to higher temperatures. The experiments next described were designed to analyse these processes that bring about the end of diapause, processes that we shall refer to as 'diapause-ending processes'.*

The larvae used were removed from their host puparium when they were firmly in diapause, i.e. at least 1 week after adult development would have been completed had growth not been arrested. *Mormoniella* were resistant to this treatment and, so far as could be judged, behaved in all respects as if still enclosed within puparia. Defaecation which just precedes the larval-pupal moult was chosen as a convenient point to mark the end of diapause.

(1) *The effects of constant low temperature*

Several thousand larvae that had been in diapause for 1 month were divided into groups of forty and placed at 2, 5, 10, 15 and 25° C. in shell vials. At 2-week intervals forty were removed from each temperature, placed at 25° C., and observed daily. The results are recorded in Figs. 2 and 3. None of the animals placed at 15 or 25° C. escaped from diapause. The termination of diapause was completed most rapidly at 2° C., where the greatest proportion of animals broke diapause after 8 weeks of chilling and where the number of days at 25° C. required for the breaking of diapause was by far the smallest. Also, as has been observed in many other species (Salt, 1947; Andrewartha, 1952), the data show that as the period of exposure to low temperature increased, diapause terminated more rapidly upon return to 25° C. and a larger fraction of the chilled larvae eventually developed. In simplest terms the diapause-ending processes are favoured by low temperature.

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* The term 'diapause development' coined by Andrewartha (1952) for these same processes was of great significance in that it drew general attention to the processes that lead to the end of diapause. But since the term might be construed to denote the processes that lead to diapause, the less ambiguous term 'diapause-ending processes' was chosen.

If the low-temperature treatment is continued beyond the 16 weeks necessary to complete the diapause-ending processes at all effective chilling temperatures, the animals continue in diapause because the early stages of pupation will not occur at temperatures below 15° C. The dormant larvae remain alive for many months at the low temperatures, but viability gradually falls if post-diapause development is prevented for more than a year. After about 2½ years mortality is almost complete.

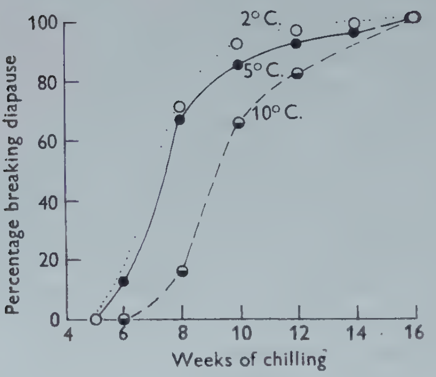

Fig. 2

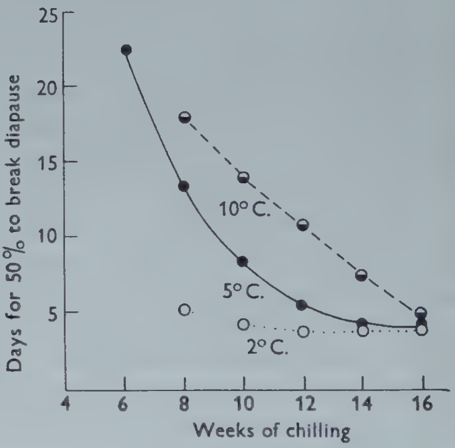

Fig. 3

Fig. 2. Effects of various periods of chilling at several temperatures on the percentage of *Mormoniella* larvae breaking diapause.

Fig. 3. The time in days for 50% of the larvae described in Fig. 2 to break diapause.

(2) *The effects of alternating temperatures*

Diapausing larvae, 2–4 weeks after they had entered diapause, were exposed to alternating periods at 5 and 25° C. according to the following schedule:

(*a*) 5° C. continuously.
(*b*) 5° C. for 7 days, 25° C. for 1 day, 5° C. for 7 days, etc.
(*c*) 5° C. for 7 days, 25° C. for 2 days, 5° C. for 7 days, etc.
(*d*) 5° C. for 7 days, 25° C. for 4 days, 5° C. for 7 days, etc.
(*e*) 5° C. for 7 days, 25° C. for 7 days, 5° C. for 7 days, etc.
(*f*) 5° C. for 2 days, 25° C. for 2 days, 5° C. for 2 days, etc.

After a cumulative exposure to 5° C. for N weeks, sixty animals were removed from each set of conditions, placed at 25° C. and their development observed. The results summarized in Fig. 4 reveal that the effects of subthreshold chilling in terminating diapause can be undone by warming. Two days at 25° C. undid a great deal of the effects of 7 days at 5° C., while 7 days at 25° C. almost completely undid the effects of 7 days at 5° C.

These results and the results obtained with constant chilling are consistent with the hypothesis that two reactions are at work (Schneiderman, 1957). One reaction is favoured by low temperature: this is the primary reaction of the diapause-ending processes which leads to the synthesis and accumulation of some substance (probably within the insect's brain). When this substance reaches threshold concentration it enables the insect, when returned to high temperature, to moult. The substance is apparently broken down at high temperatures. 'Chilling' at temperatures above 15° C. never terminates diapause, and we may conclude that at temperatures above 15° C. the rate of the breakdown reaction exceeds the rate of the synthetic reaction, so that the substance never accumulates to threshold concentrations. Below 15° C. the synthetic reaction outstrips the breakdown reaction and the substance accumulates to threshold levels. In other words, the positive temperature coefficient (Q_{10}) of the synthetic reaction is less than that of the breakdown reaction, and at about 15° C. the rates of the two reactions are equal. The following experiments were designed to test the hypothesis and to define further the synthetic and the breakdown reactions.

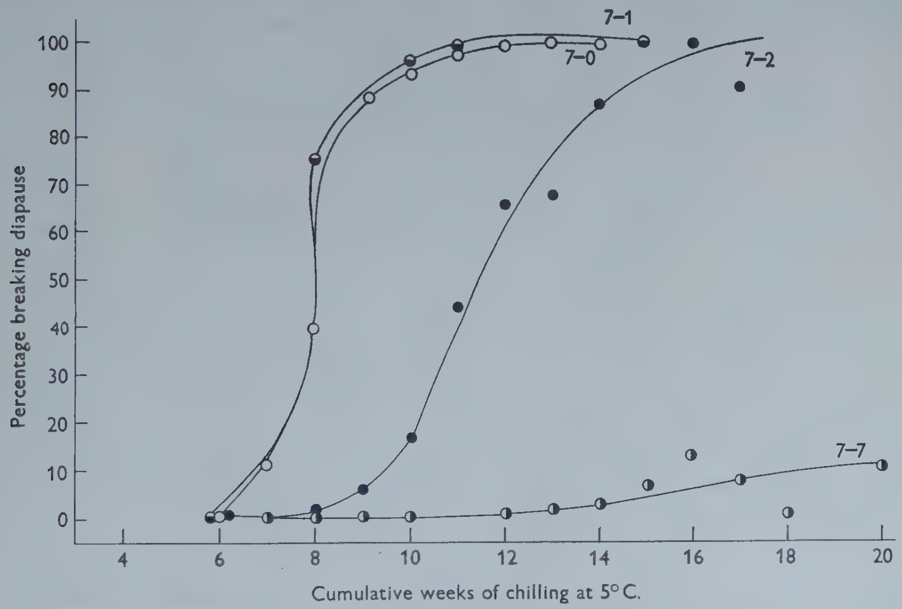

Fig. 4. Effects of alternating periods of chilling and warming in terminating diapause in *Mormoniella*. Data for 7–4 are substantially the same as those for 7–2; data for 2–2 are substantially the same as those for 7–7.

(3) *The synthetic reaction*

If low temperature acts by effectively promoting some synthetic reaction in the insect, this synthetic reaction—presumably requiring energy—ought to be inhibited when the insect's metabolism is decreased. A simple method of inhibiting metabolism during low-temperature exposure is to chill larvae at very low temperatures. To this end groups of diapausing larvae were stored at −2 to −6° C. for 6, 12 and

16 weeks. They failed to break diapause when returned to 25° C., although they did survive the chilling experience. To see whether any of the diapause-ending processes had taken place at the very low temperature, some of the larvae which had been chilled below zero for 16 weeks were given 2 and 4 additional weeks of chilling at 5° C. before being returned to 25° C. These animals also failed to break diapause when returned to 25° C. Apparently little of the diapause-ending processes took place at subzero temperatures presumably because metabolism proceeded at too slow a pace. Exposure to subzero temperatures caused no permanent damage, for when the larvae were rechilled at 5° C. for 8 or 10 weeks they developed normally.

A more effective method of lowering the insect's metabolism during chilling is to chill animals anaerobically. While this procedure is not possible with many diapausing insects which die promptly of oxygen lack, *Mormoniella* is singularly resistant to anaerobiosis. Even at 25° C. the LD 50 is about 7 days and 10% survive as long as 17 days. At low temperatures the survival was far longer as the following experiments showed. Groups of forty diapausing larvae were maintained in anaerobic flasks (see Methods) at 5° C. for periods up to 28 weeks. Parallel control groups were kept in air at 5° C. After treatment the larvae were returned to air and placed at 25° C. along with controls. As Table 9 shows, anaerobiosis at 5° C. for up to 10 weeks had little effect on the viability of the animals, and within a day of being returned to air at 25° C. they regained their motility. Exposure for 28 weeks resulted in considerable mortality, but nonetheless nearly 10% of the animals survived. Although they remained alive and apparently healthy for many months, not one of the anaerobically chilled larvae pupated, while the air-chilled control groups broke diapause and developed normally. Thus anaerobiosis prevented chilling from ending diapause. When the anaerobic animals were rechilled in air for an adequate period many initiated development and some of them developed normally, indicating that the anaerobic régime caused no permanent damage to their endocrine system. From the results of these experiments it appears that the diapause-ending processes in *Mormoniella* demand oxidative metabolism, and have a temperature optimum at about 2° C.

Table 9. *The effect of chilling in the absence of oxygen on diapausing larvae of* Mormoniella

Weeks of chilling at 5° C.	% surviving 3 weeks after return to air at 25° C.	% developing
3	100	0
8	85	0
10	85	0
16	62	0
20	26	0
28	8	0

Although the diapause-ending processes occur most rapidly at temperatures below 15° C., evidence that they may also proceed at higher temperatures is provided by the following experiments. Diapausing larvae were removed from puparia when the non-diapausing larvae of he same brood had reached the black pupal stage, i.e. the larvae had been in diapause for about 4 days. These diapausing larvae of known age were maintained at 25° C. At intervals, groups were placed at 5° C. for 6 and 8 weeks of chilling and then returned to 25° C.

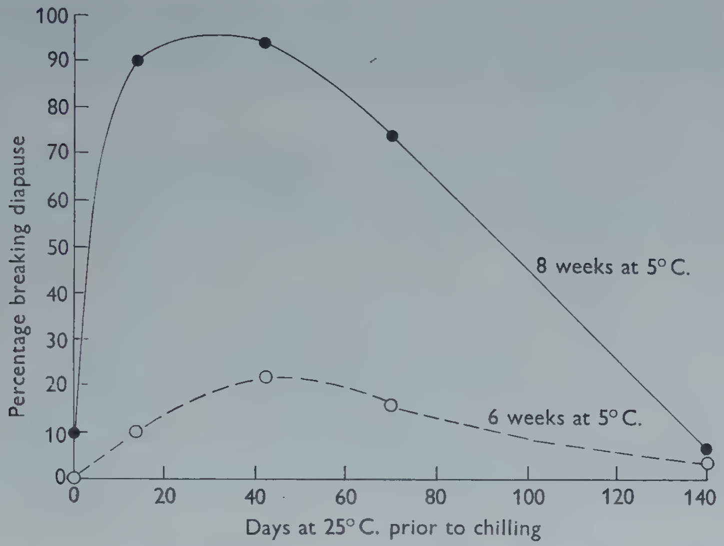

Fig. 5. Effects of incubation at 25° C. prior to chilling on the percentage of *Mormoniella* larvae breaking diapause after 6 and 8 weeks of chilling.

The results are presented graphically in Fig. 5. Incubation at 25° C. for several weeks reduced the duration of chilling necessary to break diapause, i.e. 25° C. apparently promoted the diapause-ending processes, albeit very slowly. After 10 weeks, however, this promoting effect began to wear off. These findings are consistent with the view that some diapause-ending processes can take place at high temperatures (see also Discussion).

(4) *The breakdown reaction*

To examine the nature of the breakdown reaction the following experiments were conducted. Their basis was the observation that the effects of chilling could be undone by warming and the fortunate fact that *Mormoniella* is resistant to anaerobiosis. Animals were chilled for a subthreshold period (a period too short to enable the larvae to escape from diapause). Following this, they were placed at elevated temperatures for various intervals under both aerobic and anaerobic conditions. In the first experiment of this kind, groups of twenty-five diapausing larvae, 2–4 weeks after they entered diapause, were chilled at 5° C. for 24 days, and then subjected to specific experimental conditions. After this they were returned to 5° C. for an additional 32 days of chilling (a total period of chilling of 8 weeks). Thereupon they were placed at 25° C. and their development observed. The larvae were exposed to the following experimental conditions between the two periods of chilling: incubation at 25° C. for 4, 8 or 25 days in air, or 4 or 8 days anaerobically; incubation at 30° C. for 3 days either in air or anaerobically. Control groups were placed at 25° C. after 0 day, 24 days, 7, 8, 9 or 10 weeks of continuous chilling at 5° C. The results are tabulated in Table 10A and Fig. 6. It can be clearly seen in Fig. 6, and in the table, that a brief period of aerobic warming undoes some of the effect of 24 days of chilling. Even more striking, and quite unexpectedly, the animals incubated anaerobically showed much more development than the corresponding air controls, which were continuously chilled. These results were confirmed and amplified in the following experiments.

Table 10. *The effect on diapausing larvae of* Mormoniella *of interrupting* 8 *weeks of chilling at* 5° C. *after* 24 *days by aerobic and anaerobic incubation at* 25 *or* 30° C. *for specific periods*

A

Total weeks of chilling	Conditions of incubation	% of survivors developing
7	—	12
8	—	40
9	—	83
10	—	87
8	4 days aerobic at 25° C.	4
8	4 days anaerobic at 25° C.	78
8	8 days aerobic at 25° C.	16
8	8 days anaerobic at 25° C.*	58
8	3 days aerobic at 30° C.	8
8	3 days anaerobic at 30° C.	92
8	5 days aerobic at 30° C.	9
8	25 days aerobic at 25° C.	12

B

Total weeks of chilling	Conditions of incubation	% of survivors developing	Days required for half to initiate development
3½	—	0	—
6	—	9	9
7	—	10	7
8	—	78	7
9	—	95	6
10	—	97	4
8	4 days aerobic at 25° C.	35	8
8	4 days anaerobic at 25° C.	91	4
8	8 days aerobic at 25° C.	38	7
8	8 days anaerobic at 25° C.†	62	5
8	40 days aerobic at 25° C.	15	—
8	3 days aerobic at 30° C.	43	8
8	3 days anaerobic at 30° C.	88	4
8	5 days aerobic at 30° C.	45	8

* Only 50 % survived. † Only 65 % survived.

Groups of 100 diapausing larvae had 8 weeks of chilling at 5° C., interrupted after 24 days by warming under specific experimental conditions. The several conditions and the development of the animals at 25° C. after this régime are recorded in Table 10B. It can be judged from the table that a brief interval of aerobic warming caused a measurable setback in development, while 40 days of warming had a considerable effect. Furthermore, as in the previous experiment, anaerobic warming markedly accelerated the termination of diapause. These experiments suggest that the breakdown reaction is aerobic and that the diapause-ending processes are accelerated by brief anaerobic warming.

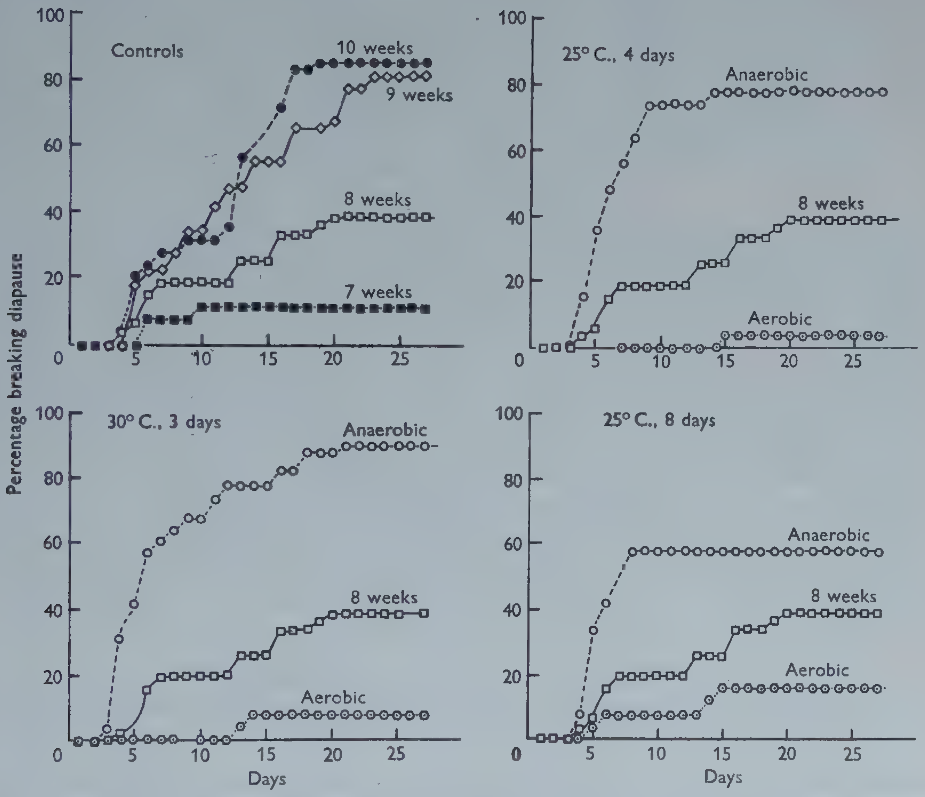

Fig. 6. Effects of interrupting 8 weeks of chilling after 24 days by warming for specific intervals under aerobic and anaerobic conditions.

Further insight into the breakdown reaction was provided by the following experiment, which was designed to ascertain whether the build-up reaction of the diapause-ending processes ever became irreversible. *Mormoniella* larvae were chilled at 5° C. for 30 weeks. This meant that the diapause-ending processes were completed and nearly 100% would break diapause when placed at 25° C. Groups of twenty-five of these larvae were given thermal shocks by exposing them to 35, 38, 40, or 45° C. for from 1 hr. to several days. The results recorded in Table 11 reveal that once the diapause-ending processes have been completed in *Mormoniella*, they cannot be undone, even by exposure to high temperature.

Table 11. *The effects of thermal shocks on the termination of diapause in chilled larvae of* Mormoniella

Temperature of shock (° C.)	Duration of shock	% remaining in diapause	% developing	% dead
25*	—	0–10	90–100	0
35	8 days	7	71	22
35	8 days	9	62	29
38	1 day	0	91	9
38	2 days	0	53	47
40	4 hr.	0	60	40
40	1 day	0	65	35
40	1 day	8	46	46
45	2 hr.	5	45	50

* Controls put directly at 25° C.

Experiments were next undertaken to ascertain when the diapause-ending processes became irreversible. Since it was usually reversible after 24 days at 5° C., groups of diapausing larvae were chilled at 5° C. for a period longer than 24 days. They were then warmed either aerobically or anaerobically and returned to 5° C. until they had received a total of 8 weeks of chilling. Thereupon they were placed at 25° C. and their development observed. The data revealed that after 38 days at 5° C., the effects of chilling could no longer be reversed by brief periods of aerobic warming. Results of a typical experiment are presented in Fig. 7.

From these several experiments it appears that the diapause-ending processes consist of at least two phases: a phase that can be reversed by brief periods of aerobic warming, which is succeeded by a phase that is substantially indifferent to aerobic warming.

(5) *Further analysis of the effects of anaerobic warming*

From the experiments considered thus far, it is not clear whether anaerobic warming resembles chilling in promoting the diapause-ending processes or is something quite distinct. Experiments were conducted to determine whether animals exposed to subthreshold chilling could be caused to break diapause by simply warming them anaerobically. Larvae were divided into groups of about fifty and put at 5° C. 3 weeks after entering diapause. The results of a typical experiment are presented in Table 12. None of the larvae chilled at 5° C. for 38 days developed when placed directly at 25° C. in air. However, if after chilling these larvae were maintained anaerobically for 4 days at 25° C., then 8% broke diapause when they were returned to air. The anaerobic treatment enabled these larvae to complete their diapause-ending processes, and in this sense it resembled chilling. The table also reveals the accelerating effects of anaerobiosis on the 45- and 57-day groups and thus confirms and extends the findings reported in the previous

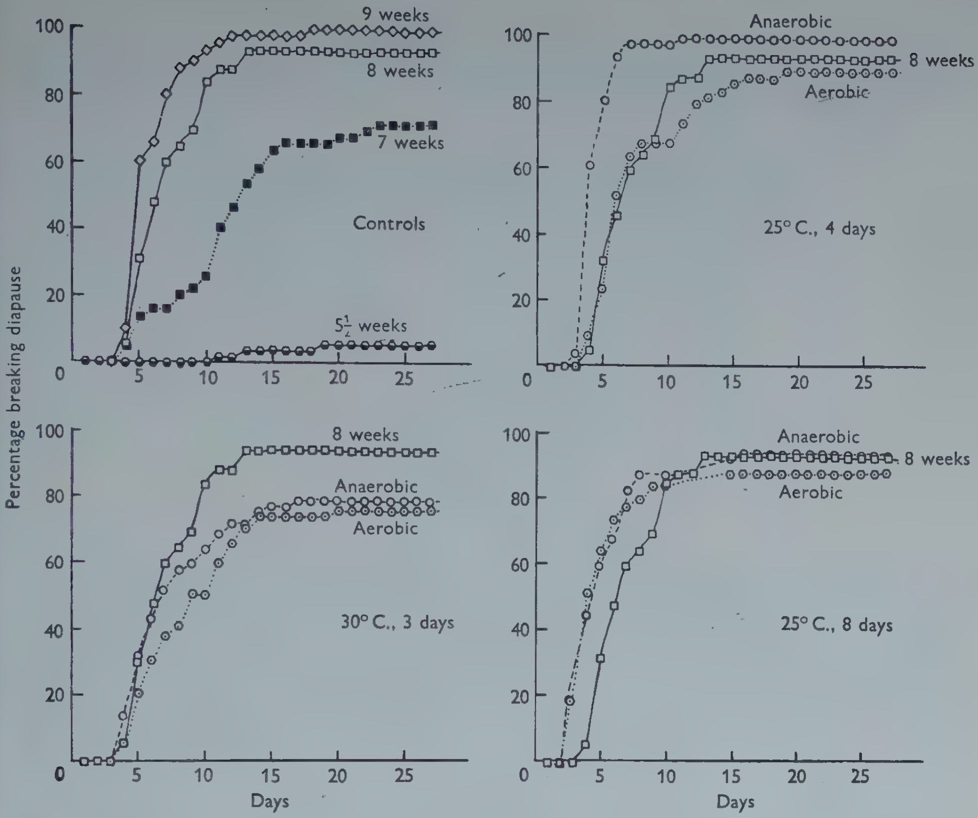

Fig. 7. Effects of interrupting 8 weeks of chilling after 38 days by warming for specific intervals under aerobic and anaerobic conditions.

Table 12. *The effect of aerobic and anaerobic incubation at* 25° C. *on diapausing larvae of* Mormoniella *chilled at* 5° C. *for specific periods*

Total days of chilling	Days at 5° C. prior to incubation	Conditions of incubation	Days at 5° C. after incubation	% of survivors developing
25	25	—	0	0
25	25	4 days anaerobic	0	0
31	31	—	0	0
31	31	4 days anaerobic	0	0
32	25	4 days air	7	0
32	25	4 days anaerobic	7	0
38	38	—	0	0
38	38	4 days anaerobic	0	8
45	45	—	0	4
45	38	4 days air	7	4
45	38	4 days anaerobic	7	28
49	49	—	0	12
56	56	—	0	60
57	25	4 days air	32	12
57	25	4 days anaerobic	32	92

Table 13. *The effect on diapausing larvae of* Mormoniella *of interrupting* 8 *weeks of chilling at* 5° C. *after* 20 *days by consecutive periods of aerobic and anaerobic incubation at* 25° C.

Total days of chilling	Conditions of incubation	% of survivors developing
49	—	0
56	—	8
70	—	94
56	4 days anaerobic	70
56	4 days air	24
56	4 days air followed by 3 days anaerobic	50
56	4 days anaerobic followed by 3 days air	16
56	4 days air followed by 3 days anaerobic followed by 4 days air	12

section. This result is consistent with the view that anaerobic warming has the same final effect on the diapause-ending processes as chilling.

If a brief period of anaerobic warming is qualitatively the same as a brief period of chilling in promoting the diapause-ending processes, then, like the effects of chilling, the effects of anaerobic warming should be reversed by a period of aerobic warming. To test this possibility, groups of fifty larvae that had been in diapause for one month were chilled at 5° C. for 20 days. Following this they were exposed to consecutive periods of aerobic and anaerobic incubation at 25° C., and they were then chilled for 35 more days. Finally, after a total of 8 weeks of chilling, they were returned to 25° C. for development. The conditions of incubation and the results are summarized in Table 13. The undoing of the subthreshold chilling by the 4 days of aerobic warming was not evident in this experiment. However, the accelerating action of 4 days of anaerobiosis was striking. Furthermore, it is clear from the table that, even after the animals had been incubated in air, anaerobiosis accelerated development. It is also evident that aerobic incubation undid the accelerating effect of anaerobiosis. In this respect anaerobic warming and aerobic chilling are similar—both are commonly reversed by aerobic warming.

TERMINATION OF DIAPAUSE IN *TRITNEPTIS*

Unlike *Mormoniella*, diapausing larvae of *Tritneptis* fail to survive for prolonged periods when removed from the cocoons of their host. Indeed, only 12 weeks of chilling at temperatures of 2–10° C. killed nearly half the isolated larvae, but there was little mortality among larvae chilled while still enclosed within their host cocoons. Sufficient experiments were conducted on both isolated and enclosed larvae of *Tritneptis* to demonstrate that exposure to low temperature terminates larval diapause much as it does in *Mormoniella*. Results of a typical experiment on isolated larvae are recorded in Table 14. It is also noteworthy that occasional individuals developed after several months at 15° C., indicating that the diapause-ending processes have a higher threshold temperature in *Tritneptis* than in *Mormoniella*.

Table 14. *The effect of chilling at 5° C. for various periods on the termination of larval diapause in* Tritneptis

Weeks of exposure to 5° C.	% developing of those which survived for 2 weeks after being returned to 25° C.
5	4
6	5
7	24
8	50
9	45
10	100

DISCUSSION

(1) *The induction of diapause*

In the case of *Mormoniella* the experimental results fail to confirm the assertions of Cousin (1932) that diapause results from deficient larval nutrition, and of Van der Merwe (1945) that diapause is induced in larvae exposed to low humidity or falling temperatures. But they leave little doubt that exposing female wasps to low temperature during oögenesis causes their offspring to enter diapause at the end of the last larval instar. In some way the temperature stimulus appears to be communicated from parent to offspring through the egg. The low-temperature stimulus may be acting directly on the ovaries and thereby affecting the chemical composition of the egg prior to the initiation of cleavage. Or the stimulus may be acting on some other organ within the insect and affecting indirectly the composition of the egg.

A decision between these alternatives will have to await appropriate ovarian transplantation experiments. In any event, low temperature causes the female to lay an egg that is qualitatively different from an ordinary egg in that the larva emerging from it eventually enters diapause. It is also clear that the low-temperature effect wears off after several days and chilled females return to producing non-diapausing offspring. Since some females produce mixed broods for several days after chilling, the effect is not all-or-none as far as the female is concerned. Conceivably it is all-or-none for the eggs within one of the eight ovarioles, but it is more likely that there is a distribution of sensitivity among the eggs of a given female. Moreover, since some females produce a proportion of diapause offspring without any chilling, there is also a distribution of susceptibility to diapause induction among females. Some females are much more sensitive to temperature than others. In this latter connexion, it is worth noting that Moursi (1946*b*) failed to obtain any diapause in the offspring of female *Mormoniella* that had been chilled as pupae or adults. However, his experiments were conducted on wasps collected in southern California, and it is not unlikely that genetic differences between his population and the Woods Hole wild-type used in our experiments are at least in part responsible for the apparent different susceptibilities of the females to diapause induction.

So far as we are aware, only a few examples are known of maternal stimuli being transmitted to the larval progeny (Simmonds, 1948; Cragg & Cole, 1952; Lees, 1955). The closest parallel to *Mormoniella* is found in the pteromalid *Spalangia drosophilae*. This pupal ectoparasite of the frit-fly (*Oscinella frit*) undergoes a facultative larval diapause in the third instar when the animals are reared continuously at temperatures below 24° C. (Simmonds, 1948). Furthermore, the incidence of diapause increases in the offspring of senile females. Simmonds concludes that '...the temperature to which a female has been subject during development and temperature changes prior to the commencement of oviposition affect the potentialities with regard to diapause of eggs laid subsequently. The temperature of development of eggs within the ovarioles...has some affect on the quality of the eggs and their potentialities for future development' (p. 401). These conclusions seem to apply to *Mormoniella*.

Although the present experiments provide few clues about how low temperature acting on the parent has a delayed action in producing diapause in the offspring after several moults, they establish that the phenomenon is real. Moreover, they suggest that the generally held view that 'the central nervous system is the vehicle which carries and eventually transmits the "directions" of the environment' in inducing larval and pupal diapause may be inadequate to explain some kinds of facultative diapause (Lees, 1955, p. 114). In the case of *Mormoniella*, it is difficult to regard low temperature as a 'token' stimulus which acts via the central nervous system (Lees, 1955, p. 31). More probably, as mentioned above, low temperature directly affects the metabolism of the developing egg or some organ that contributes to the substance of the egg. It is possible that this organ might be an endocrine organ within the female wasp that secretes a 'diapause hormone'. The production of such a hormone by the suboesophageal ganglion of the commercial silkworm causes the eggs in the ovaries to become 'diapause eggs', i.e. embryos developing from these eggs enter diapause at the very young germ-band stage (Fukuda, 1951; Hasegawa, 1951). If such a mechanism operates in *Mormoniella*, then low temperature might be acting as a token stimulus via the nervous system of the mother.

The mechanism of diapause induction in *Tritneptis* is far easier to explain than that of *Mormoniella*. In *Tritneptis*, to be effective, low temperature must act on the larva itself to produce diapause in that generation.

It is noteworthy that two wasps as closely related as *Mormoniella* and *Tritneptis* should use very different mechanisms to induce diapause. This fact supports the suggestion of Lees (1955) that diapause has frequently been evolved independently in closely related groups. In this connexion, attempts were made to induce diapause in a third chalcid in the family Eulophidae, *Dahlbominus fuscipennis** (=*Microplectron fuscipennis*), by the stimuli which proved effective for *Mormoniella* and *Tritneptis*. None of the stimuli was effective, although *Dahlbominus* has been reported to have a facultative larval diapause (Morris & Cameron, 1935). Possibly, factors other than temperature are critical in this insect.

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The arguments up to this point have not required any decision about the immediate cause of larval diapause in *Mormoniella* and *Tritneptis*. However, there is considerable evidence which has been summarized elsewhere (Lees, 1955, pp. 108–10, 1956; Church, 1955; Williams, 1956; Schneiderman, 1957) that the larval and pupal diapause of the Lepidoptera and Hymenoptera usually results from the inactivation of specific neurosecretory cells in the insect's brain. These cells normally produce and release a tropic factor, the brain hormone, which stimulates the prothoracic glands to secrete the growth and moulting hormone. When the neurosecretory cells cease producing their hormone, growth and moulting cease and diapause supervenes. Under this view, the larval diapause of *Mormoniella* and *Tritneptis* would stem from the inactivation of the larval neurosecretory cells. While this is probably true, we ought not to forget that diapause has probably evolved independently many times and, in the absence of appropriate surgical experiments, we should not completely rule out the possibility that inactivation of some link in the endocrine chain other than the brain may be the central cause of diapause in some insects (cf. for example, Ozeki (1954) and Rahm (1952)).

(2) *The termination of diapause*

Since only a few experiments were conducted on the termination of diapause in *Tritneptis*, this discussion will focus on *Mormoniella*. The original hypothesis to explain diapause-ending processes that furnished a starting point for the present experiments proposed two competing reactions with different positive temperature coefficients—a synthetic reaction and a breakdown reaction (cf. p. 535). Indeed, this has been the most favoured hypothesis to explain the action of low temperature in terminating diapause and has been suggested by numerous workers on various grounds (Andrewartha, 1943, 1952; Salt, 1947; Williams, 1947; Lees, 1955, 1956). The experimental results demonstrate the following about these two postulated reactions.

The synthetic reaction

(1) The synthetic reaction is in part obligatorily aerobic, for chilling fails to exert an effect unless it is performed aerobically in at least the initial stages. Thus larvae chilled anaerobically for many months, although they survived, failed to develop at 25° C. unless rechilled in air.

(2) The synthetic reaction can proceed in part in the absence of oxygen, for there was a marked increase in the proportion of animals that break diapause, and a decrease in the time required for the initiation of development when larvae were warmed anaerobically for a brief interval during the period of chilling. Furthermore, when larvae were chilled for a subthreshold period, a brief interval of anaerobic warming sufficed to enable some of the larvae to terminate diapause. This is consistent with the view that anaerobic warming and aerobic chilling favour the synthetic reaction.

(3) The temperature optimum for the aerobic phase of the synthetic reaction is about 2° C., and little of the diapause-ending processes occurs at temperatures below −6° C. or above 15° C. (cf. Lees, 1953, p. 476). Thus, in *Mormoniella* as in the eggs of the grasshopper *Austroicetes* (Andrewartha, 1943) the temperature ranges for the diapause-ending processes and for morphogenesis do not overlap, and the two cannot take place concurrently. This is in contrast to pupae of the *cecropia* silkworm (Williams, 1956) and to eggs of *Melanoplus* and *Locusta* (Andrewartha, 1943).

(4) Although diapause will never be terminated by keeping larvae continually at temperatures above 15° C., diapause-ending processes do take place slowly at these temperatures. Thus a period of exposure to temperatures above 15° C. *prior to chilling* decreased the amount of chilling necessary to terminate diapause. This finding is in agreement with the observations of Emme (1949) on the termination of diapause in the eggs of *Bombyx* and of Church & Salt (1952) in the eggs of *Melanoplus*.

An alternative explanation of this finding is that exposure to low temperature is not effective until the last instar *Mormoniella* larva has reached some definite stage of physiological development and that several weeks are required after the last larval moult for these changes to take place (cf. Andrewartha, 1943). But inasmuch as there is other evidence that diapause-ending processes can occur at high temperatures (see below) the explanation offered in the preceding paragraph seems more attractive. In any event the results emphasize that keeping diapausing larvae at 25° C. prior to chilling is not an indifferent procedure and must be taken into account in all experiments on diapause termination. They also make it clear that a diapausing larva at 25° C. is not in *status quo* but is undergoing slow but persistent physiological changes.

(5) The synthetic reaction can be completed in part at 25° C. *after chilling*. For when larvae are chilled for 6 weeks at 5° C., more than 12 days are required for those that initiate development to do so, while after 14 weeks of chilling development is initiated within 4 days. In the 6-week group, the synthetic reaction presumably was completed during the 12 days at 25° C. (cf. Browning, 1952, p. 352).

The breakdown reaction

(1) The breakdown reaction is apparently aerobic, for the effects of chilling seem to be undone only by aerobic warming.

(2) Part of the synthetic reaction becomes irreversible after about a month of chilling, for brief aerobic warming fails to exert an effect if it intervenes after the larvae have been exposed for more than a month to 5° C. Thus, part of the synthetic reaction becomes irreversible long before the diapause-ending processes are completed. In this respect *Mormoniella* behaves quite differently from diapausing prepupae of the saw-fly *Cephus cinctus*, in which the synthetic reaction can be reversed by exposure to high temperature apparently after the diapause-ending processes have been completed (Salt, 1947; Church, 1955). The fact that prolonged aerobic warming undoes a considerable proportion of the effect of subthreshold

chilling is of interest when considered in relation to species which have a long-enduring diapause of several years in nature (e.g. as long as 6 years for some prepupae of the saw-fly *Gilpina polytoma* and up to 12 years for larvae of the wheat-blossom midge *Sitodiplosis mosellana* (cited by Lees, 1955)). Apparently in these species the amount of the diapause-ending processes completed in one winter is below threshold. It is likely that much of the net effect of the diapause-ending processes is lost before the succeeding winter, but not all of it, so that some residuum is carried over from one winter to the next until a threshold is reached (cf. Lees, 1955, p. 60).

(3) Aerobic warming undoes the accelerating effects of anaerobic warming as well as the effects of aerobic chilling. This is consistent with the view that anaerobic warming and aerobic chilling effectively promote the same synthetic reaction.

The experiments provide no evidence for the site of action of low temperature. However, as has been pointed out elsewhere (Schneiderman, 1957), there is considerable evidence that in most diapausing Lepidoptera and Hymenoptera low temperature acts directly on the brain, without the intervention of the rest of the central nervous system and probably of the rest of the body. Under this view, diapause-ending processes involve reactions occurring within the brain of the insect.

It must be pointed out, however, that the criterion we have used to mark the end of diapause—namely, defaecation—involves many reactions besides the diapause-ending processes that we propose occur within the insect's brain. In addition to these processes, there is the actual secretion into the blood of the brain hormone, the activation of the prothoracic glands by the brain hormone and the secretion of the prothoracic gland hormone, and, finally, the interaction of the prothoracic gland hormone with the tissues of the insect. In our opinion, the effects of low temperature and of aerobic and anaerobic warming on the termination of diapause result primarily from the action of these agencies on processes occurring within the insect's brain. This does not, of course, gainsay the possibility that some of these factors might be acting on other processes. Thus it is conceivable, for example, that anaerobic warming might in some way increase the sensitivity of the prothoracic glands to the brain hormone. However, in the absence of evidence to the contrary, we prefer to think of these several agencies as acting primarily on the brain itself.

Our results do not permit us to pinpoint any specific reactions within the insect's brain, but they do enable us to rule out certain hypotheses, for example, the early proposals of Andrewartha (1952) and Andrewartha & Birch (1954) (cf. Schneiderman, 1957). They also rule out the simple scheme of two reactions with different temperature coefficients, which has served as a working hypothesis for this and other studies (Williams, 1956; Schneiderman, 1957). Clearly, the synthetic reaction must be complex, having both aerobic and anaerobic phases, a phase that is favoured by low temperature but is reversible, followed by a phase that is also favoured by low temperature but is irreversible. Actually, it would seem more appropriate to think in terms of *synthetic reactions* (cf. Lees, 1955, p. 64). It is notable that, while there are evidently several synthetic reactions involved in the diapause-ending processes, there is no evidence for multiple breakdown reactions.

It is likely that the various synthetic reactions lead to the synthesis of the same active substance within the insect's brain—a substance that is necessary for the production or release of the brain hormone. These synthetic reactions are opposed by an oxidative breakdown reaction. Low temperature acts by slowing down this breakdown reaction within the brain, thereby enabling the brain to accumulate the substance. The nature of the substance that accumulates within the chilled larval brains is of uncommon interest. If we make the assumption that at low temperatures the same substance is being synthesized in these larval brains as in the pupal brains of Lepidoptera, then it is probable that the substance is acetyl choline. For Van der Kloot (1955) has shown that chilling promotes the synthesis of a cholinergic substance within the diapausing pupal brain of the *cecropia* silkworm, *Hyalophora cecropia*, and this invariably precedes the activation of the neurosecretory cells within the brain. Under this view, the breakdown reaction is either the breakdown of acetyl choline or the removal of one of the intermediates in its path of synthesis. Since the breakdown reaction seems to be aerobic it is probably enzymatic and not a simple non-enzymatic breakdown of acetyl choline as has been suggested (Williams, 1956). The apparent oxygen requirement of the breakdown reaction also rules out the possibility that the reaction is the simple hydrolysis of acetyl choline (Schneiderman, 1957). It seems likely that specific descriptions of the chemical reactants involved in both the synthetic and breakdown reactions will have to await further direct study of the metabolism of the insect brain.

SUMMARY

1. Experiments have been conducted to determine the extrinsic factors that cause facultative diapause in two parasitic chalcid wasps, *Mormoniella vitripennis* and *Tritneptis klugii*, and to analyse the mechanism of diapause termination.

2. In both species diapause occurs in the last larval instar after the feeding period has ended and just prior to defaecation. The diagnostic feature of the diapausing larva is that it does not immediately moult into a pupa.

3. In *Mormoniella* exposing females to low temperature during oögenesis causes their progeny to enter diapause at the end of the last larval instar. Low temperature thus causes the female to lay an egg that is qualitatively different from an ordinary egg in that the larva emerging from it eventually enters diapause. This action of low temperature on the female wears off after several days and the wasp returns to producing non-diapausing offspring.

4. In *Tritneptis* low temperature also produces diapause, but in this species low temperature, to be effective, must act on the larva itself between the second and final instar to produce diapause in that generation.

5. The diapause of *Mormoniella* was considered in relation to maternally induced diapause in other species and two possible mechanisms for the action of low temperature were suggested, namely, a direct action on the ovaries or an indirect action through the maternal production of a diapause hormone.

6. It was found that exposure to low temperatures enables larvae of both species to break diapause and complete their development when subsequently placed at 25° C.

7. The mechanism of action of low temperature in terminating diapause was examined in *Mormoniella* by exposing larvae to various temperature régimes in the presence and absence of oxygen.

8. Ten weeks at 5° C. enabled nearly 90% of the larvae to terminate diapause when returned to 25° C.; after 6 weeks at 5° C. less than 10% developed. Chilling at 2° C. was more effective than 5 or 10° C., while temperatures above 15 or below −6° C. were ineffective.

9. Although diapause was never terminated by keeping larvae continually at temperatures above 15° C., a period of exposure to temperatures above 15° C. *prior to chilling* decreased the amount of chilling necessary to terminate diapause.

10. Larvae chilled in the absence of oxygen for as long as 28 weeks failed to break diapause but developed when subsequently rechilled in air.

11. After receiving a *threshold* exposure to low temperature larvae could not be returned to diapause by temperature shocks as high as 45° C.; however, the effects of *subthreshold* chilling were reversed by exposure to 25° C. Thus, animals chilled for a total of 20 weeks, with 1 week of warming after each week of chilling, failed to develop. Similarly, it was found that interrupting 8 weeks of chilling on the 25th day by 4 days of warming partially undid the chilling.

12. When warming was conducted in the absence of oxygen it failed to undo the effects of subthreshold chilling and the termination of diapause was markedly accelerated. Indeed, when larvae were chilled for a subthreshold period, a brief interval of anaerobic warming sufficed to enable some of the larvae to terminate diapause.

13. In addition to the above, a variety of other experiments were conducted with alternating periods of chilling and warming in the presence and absence of oxygen. These led to a hypothesis which seems to account for the action of low temperature in terminating larval diapause.

14. The hypothesis focuses attention on the neurosecretory cells of the insect's brain and suggests that low temperature slows down an aerobic breakdown reaction within the larval brain and permits the synthesis of a substance necessary for neurosecretory activity. The initial stages of the synthetic reaction are aerobic but later stages are favoured by anaerobic warming. The nature of these reactions was discussed.

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REFERENCES

ALTSON, A. M. (1920). The life history and habits of two parasites of blow flies. *Proc. Zool. Soc. Lond.* Pt. 3, 195–243.

ANDREWARTHA, H. G. (1943). Diapause in the eggs of *Austroicetes cruciata* Sauss. (Acrididae) with particular reference to the influence of temperature on the elimination of diapause. *Bull. Ent. Res.* **34**, 1–17.

ANDREWARTHA, H. G. (1952). Diapause in relation to the ecology of insects. *Biol. Revs.* **27**, 50–107.

ANDREWARTHA, H. G. & BIRCH, L. C. (1954). *The Distribution and Abundance of Animals.* Chicago, Ill.: The University of Chicago Press.

BROWNING, T. O. (1952). On the rate of completion of diapause development at constant temperatures in eggs of *Gryllulus commodus* Walker. *Aust. J. Sci. Res.* B, **5**, 344–53.

CHURCH, N. S. (1955). Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. (Hymenoptera: Cephidae). *Canad. J. Zool.* **33**, 339–69.

CHURCH, N. S. & SALT, R. W. (1952). Some effects of temperature on development and diapause in the eggs of *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae). *Canad. J. Zool.* **30**, 173–84.

COUSIN, G. (1932). Étude expérimentale de la diapause des insects. *Bull. biol.* Supplement XV, 1–341.

COUSIN, G. (1933). Étude biologique d'un chalcidien *Mormoniella vitripennis* (Walker). *Bull. biol.* **67**, 371–400.

CRAGG, J. B. & COLE, P. (1952). Diapause in *Lucilia sericata* (Mg.) Diptera. *J. Exp. Biol.* **29**, 600–4.

EMME, A. M. (1949). Cited in Lees, 1955.

EVANS, A. C. (1933). Comparative observations on the morphology and biology of some hymenopterous parasites of carrion-infesting Diptera. *Bull. Ent. Res.* **24**, 385–405.

FERSCHL, SR. M. JOHN BAPTIST & WOLSKY, A. (1956). Observations on pigment development in pupae of *Mormoniella vitripennis* Walker. *Anat. Rec.* **125**, 599–600.

FIESER, L. (1924). A new absorbent for oxygen in gas analysis. *J. Amer. Chem. Soc.* **46**, 2639–47.

FLANDERS, S. E. (1944). Diapause in parasitic Hymenoptera. *J. Econ. Ent.* **37**, 408–11.

FUKUDA, S. (1939). Biology of *Mormoniella vitripennis* (Wlk.), a parasite of *Sturmia sericariae* Corn. (In Japanese.) *Zool. Mag.* **51**, 185–94. (Summary *Rev. Appl. Ent.*, A, **27**, 443.)

FUKUDA, S. (1951). Production of the diapause eggs by transplanting the subesophageal ganglion in the silkworm. *Proc. imp. Acad. Japan*, **27**, 672–7.

GIRAULT, A. A. & SAUNDERS, G. E. (1909). The chalcidoid parasites of the common house or typhoid fly (*Musca domestica* L.) and its allies. *Psyche, Camb., Mass.*, **16**, 119–32.

GIRAULT, A. A., & SAUNDERS, G. E. (1910). The chalcidoid parasites of the common house or typhoid fly (*Musca domestica* L.) and its allies. *Psyche, Camb., Mass.*, **17**, 9–28.

GRAHAM-SMITH, G. S. (1919). Further observations on the habits and parasites of common flies. *Parasitology*, **11**, 347–84.

HASEGAWA, K. (1951). Studies in voltinism in the silkworm, *Bombyx mori* L., with special reference to the organs concerning determination of voltinism (a preliminary note). *Proc. imp. Acad. Japan*, **27**, 667–71.

HEWITT, C. G. (1912). The large larch sawfly (*Nematus erichsonii*). *Bull. Canad. Dept. Agric.* **10**, (Series 2). *Ent. Bull.* **5**, 28–30.

JACOBI, E. F. (1939). Über Lebensweise, Auffinden des Wirtes und Regulierung der Individuenzahl von *Mormoniella vitripennis* Walker. *Archives néerl. Zool.* **3**, 197–282.

LEES, A. D. (1953). Environmental factors controlling the evocation and termination of diapause in the fruit tree red spider mite *Metatetranychus ulmi* Koch (Acarina: Tetranychidae). *Ann. Appl. Biol.* **40**, 449–86.

LEES, A. D. (1955). *The Physiology of Diapause in Arthropods.* Cambridge University Press.

LEES, A. D. (1956). The physiology and biochemistry of diapause. *Ann. Rev. Ent.* **1**, 1–16.

MORRIS, K. R. S. & CAMERON, E. (1935). The biology of *Microplectron fuscipennis*. *Bull. Ent. Res.* **26**, 407–18.

MOURSI, A. A. (1946*a*). The effect of temperature on the sex ratio of parasitic Hymenoptera. *Bull. Soc. Fouad Ent.* **30**, 21–37.

MOURSI, A. A. (1946*b*). The effect of temperature on development and reproduction of *Mormoniella vitripennis* (Walker). *Bull. Soc. Fouad Ent.* **30**, 39–61.

OZEKI, K. (1954). Cited in Lees (1956).

PARKER, H. L. (1924). Recherches sur les formes Postembryonnaires des Chalcidiens. *Ann. Soc. ent. Fr.* **93**, 268–379.

PARKER, H. L. & THOMPSON, W. R. (1928). Contribution à la biologie des Chalcidiens entomophages. *Ann. Soc. ent. Fr.* **97**, 425–65.

RAHM, U. H. (1952). Cited in Lees (1956).

RAY, D. T., HUNTER, F. & STEPHENS, G. J. (1954). Sex difference and oxygen consumption in the wasp *Mormoniella*. *Rec. Genet. Soc. Amer.* **23**, 62.

ROBERTS, R. A. (1935). Some North American parasites of blow flies. *J. Agric. Res.* **50**, 479–94.

ROUBAUD, E. (1917). Observations biologiques sur *Nasonia brevicornis* Ashm., Chalcidide parasite des pupes Muscides. *Bull. sci. Fr. Belg.* **50**, 425–39.

SALT, R. W. (1947). Some effects of temperature on the production and elimination of diapause in the wheat stem sawfly, *Cephus cinctus* Nort. *Canad. J. Res.* D **25**, 66–86.

Schneiderman, H. A. (1957). *Onset and Termination of Insect Diapause. Physiological Triggers*, T. H. Bullock, ed., pp. 46–59. Baltimore, Md.: Waverly Press, Inc.

Schneiderman, H. A. & Williams, C. M. (1952). The terminal oxidases in diapausing and non-diapausing insects. *Anat. Rec.* **113**, 55–6.

Schneiderman, H. A., Horwitz, J. & Kurland, C. G. (1956). An analysis of the action of low temperature in terminating the diapause of *Mormoniella. Anat. Rec.* **125**, 557.

Schneiderman, H. A., Kuten, J. & Horwitz, J. (1956). Effects of X-irradiation on the post-embryonic development of a chalcid wasp. *Anat. Rec.* **125**, 625–6.

Simmonds, F. J. (1948). The influence of maternal physiology on the incidence of diapause. *Phil. Trans.* B, **233**, 385–414.

Tiegs, O. W. (1922). Researches on insect metamorphosis. I. On the structure and post-embryonic development of a chalcid wasp, *Nasonia*. II. On the physiology and interpretation of the insect metamorphosis. *Trans. Roy. Soc. S. Aust.* **46**, 319–527.

Van der Kloot, W. G. (1955). The control of neurosecretion and diapause by physiological changes in the brain of the Cecropia silkworm. *Biol. Bull. Woods Hole*, **109**, 276–94.

Van der Merwe, J. S. (1945). Investigations on the biology and ecology of *Mormoniella vitripennis* Walk. (Pteromalidae, Hym.). *J. Ent. Soc. S. Afr.* **6**, 48–64.

Whiting, P. W. (1949). Mutant types in *Mormoniella. Rec. Genet. Soc. Amer.* **18**, 120.

Whiting, P. W. (1955*a*). A parasitic wasp and its host for genetic instruction and for biology courses. *Carol. Tips*, **18** (4), 13–16.

Whiting, P. W. (1955*b*). Study of genetics and host-parasite relationships (using *Mormoniella* and *Sarcophaga*). *Publ. Carol. Biol. Supply Co.* Elon, N.C., U.S.

Whiting, P. W. (1955*c*). *Mormoniella* notes: adaptation in time of eclosion, emergence and mating. *Carol. Tips*, **18** (9), 34.

Whiting, P. W. (1955*d*). Some interactions of mutant eye-color factors in the wasp *Mormoniella. Proc. Pa. Acad. Sci.* **29**, 242–5.

Williams, C. M. (1947). Physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia. Biol. Bull. Woods Hole*, **93**, 89–98.

Williams, C. M. (1956). The physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull. Woods Hole*, **110**, 201–18.

NITROGENOUS EXCRETION IN CROCODILES

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INTRODUCTION

Animals in general are known to fall into three main groups, ammonoteles, ureoteles or uricoteles according to whether ammonia, urea or uric acid is the main nitrogenous excretory metabolite in the urine. Among reptiles, Lacertilia and Ophidia are known to be uricoteles, chelonian tortoises were found to be ureo-uricoteles (Khalil & Haggag, 1955). Crocodilia—represented by alligators, crocodiles and gavials—is the least-studied reptilian order in respect of its nitrogenous excretion. The data available in the literature are those of Lewis (1918), Hopping (1923) and Coulson, Hernandez & Brazda (1950) on the analysis of urinary excreta of *Alligator mississippiensis*. These authors claim that alligators are ammonoteles. The only work on crocodiles, as the present authors can ascertain, was done by Moore as early as 1851 on *Crocodylus vulgaris* (Winterstein, 1924), but the urine sample he examined was not fresh, since it was taken from an animal 36 hr. after death and was analysed 30 days later, by which time it had a very bad smell. Moore stated that the urine contained a white precipitate of ammonium urate and that urea was absent.

The present work was undertaken on *C. niloticus* to extend knowledge on nitrogenous excretion in Crocodilia.

MATERIAL AND METHODS

Crocodylus niloticus (*Laurenti*) is an amphibious reptile living on the banks of the Nile in the Sudan. It is a very savage carnivore and great difficulties were encountered in transporting it by plane from Khartoum to Cairo; for this reason only three animals were investigated in the present work.

These animals were observed to give their nitrogenous excreta partly as liquid urine and partly as amorphous white thread-like deposits, and so both portions were analysed. Procedures for collection and analysis of both urinary portions are similar to those used by the present authors in tortoises (Khalil & Haggag, 1955).

RESULTS AND DISCUSSION

One urine sample was analysed from animals 1 and 3, and five successive urine samples were analysed from animal 2 (Table 1). Three urinary deposits were analysed from the three animals, that of animal 2 is formed of five successive samples added together (Table 2).

Table 1. *Non-protein nitrogen constituents of urine of* Crocodylus niloticus

Constituents	Nitrogen in 100 ml. urine (mg.)								
	Animal (1, ♂)	Animal (2, ♂)						Animal (3, ♂)	Average
		Five successive samples					Average		
Total N	344·00	222·70	243·60	220·70	250·00	246·20	236·64	314·90	298·50
Ammonia N	228·70	125·30	165·60	130·90	156·30	192·80	154·20	217·50	200·10
Urea N	65·61	53·53	45·24	40·11	23·66	34·26	39·35	5·22	36·74
Uric acid N	7·40	6·00	0·28	0·58	4·56	2·43	2·77	10·93	7·03
Undetermined N	42·29	37·87	32·48	49·11	65·48	16·71	40·32	81·25	54·63
	N-partition, % of total N excreted								
Total N	100	100	100	100	100	100	100	100	100
Ammonia N	66·48	56·24	67·98	59·29	62·52	78·30	64·86	69·08	66·81
Urea N	19·07	24·03	18·57	18·18	9·46	13·92	16·83	1·66	12·52
Uric acid N	2·15	2·69	0·11	0·26	1·80	0·99	1·17	3·47	2·26
Undetermined N	12·30	17·04	13·34	22·27	26·22	6·79	17·14	25·79	18·41

Table 2. *Analysis of urinary deposits of* Crocodylus niloticus (*in* 1 *g. deposit*)

Animal no. ...	1		2		3		Average	
Components ...	As N	As % to T.N.	as N	As % to T.N.	As N	As % to T.N.	As N	As % to T.N.
Total N (T.N.)	354·20	100	250·20	100	290·90	100	298·40	100
Water soluble N	96·65	27·29	95·02	37·97	108·90	37·44	100·19	34·23
Ammonia N	17·15	4·95	17·90	7·9	17·16	5·90	17·40	5·98
Urea N	0	0	0	0	0	0	0	0
Uric acid N	80·02	22·59	70·22	28·06	88·94	30·58	77·72	27·08
Water insoluble N	257·55	72·71	155·50	62·03	182·00	62·66	198·21	65·77
Uric acid N	211·00	59·61	156·10	62·40	182·00	62·58	183·06	61·53

The samples of liquid urine ranged from 3 to 10 ml. in volume. Each sample was accompanied by an amount of urinary deposit ranging from about 0·1 to 0·3 g. There was no apparent relation between the volume of urine and the weight of deposit associated with it. The pH of the liquid urine was from 7·5 to 8·5 in the samples examined.

The average value of the total nitrogen of the liquid urine in the three animals examined was 298·5 mg./100 ml. Of this value 66·81% was as ammonia, 12·52% as urea and 2·26% as uric acid.

On the other hand, it is clear from table 2 that uric acid nitrogen represents the main nitrogenous component as it formed 88·61% of the total nitrogen of the deposit. The water soluble portion of the deposit consists of ammonia and uric acid in the proportion required for the formation of acid ammonium urate.

If one considers the results in Table 1, without taking into consideration the data of the deposits in Table 2, it would certainly appear that *C. niloticus* is a typical ammonotele excreting ammonia as the main nitrogenous excretory metabolite, this substance forming 66·81% of the excretory nitrogen of the liquid urine, while urea and uric acid formed only 12·52 and 2·26%, respectively. In this respect,

Table 3. *Approximate calculation of the total excretory nitrogen (in the liquid urine + deposit) of* Crocodylus niloticus

Animal no.	Approx. vol. of urine (ml.)	Approx. wt. of deposit (g.)	Nitrogenous constituents for each collection						
			Total N.			Ammonia N			
			Liquid (mg.)	Deposit (mg.)	Total (mg.)	Liquid (mg.)	Deposit (mg.)	Total	
								In mg.	As % T.N.
1	3·5	0·2	12·0	70·8	82·8	8·0	3·4	11·4	13·8
2*	3·2	0·1	7·1	25·0	32·1	4·0	1·8	5·8	18·1
	10·0	0·1	24·4	25·0	49·4	16·6	1·8	18·4	37·2
	10·5	0·3	23·2	75·1	98·3	13·7	5·4	19·1	19·4
3	10·0	0·1	31·5	29·1	60·6	21·7	1·7	23·4	38·6
Av. % to T.N.			—	—	—	—	—	—	25·4

Animal no.	Approx. vol. of urine (ml.)	Approx. wt. of deposit (g.)	Nitrogenous constituents for each collection							
			Urea N				Uric acid N			
			Liquid (mg.)	Deposit (mg.)	Total		Liquid (mg.)	Deposit (mg.)	Total	
					In mg.	As % of T.N.			In mg.	As % T.N.
1	3·5	0·2	2·3	0	2·3	2·8	0·3	58·2	58·5	70·7
2*	3·2	0·1	1·7	0	1·7	5·3	0·2	22·6	22·8	71·0
	10·0	0·1	4·5	0	4·5	9·1	Trace	22·6	22·6	45·7
	10·5	0·3	4·2	0	4·2	4·3	0·1	67·9	68·0	69·2
3	10·0	0·1	0·5	0	0·5	0·8	1·1	27·1	28·2	85·9
Av. % to T.N.			—	—	—	4·5	—	—	—	68·5

* Only the first three of the five successive samples were taken into account, as we are not certain of the wei of deposit in the other two.

C. niloticus would be similar to its cousin, *Alligator mississippiensis* which according to Hopping (1923) excretes 75·5 % of its excretory nitrogen in the liquid urine as ammonia, while urea and uric acid represent only 4·1 and 11·4 %, respectively. Coulson *et al.* (1950) came to a similar conclusion since they found that the liquid urine of alligators contains 79·1 and 3·7 % of the total nitrogen as ammonia and uric acid, respectively, with traces of urea.

Since it was not possible to make accurate quantitative collections of both liquid urine and solid deposit, the distribution of nitrogen in the whole urine (liquid + solid) can only be given approximately. From Table 3 it can be seen that for the whole urine the figures are: ammonia, 25·4 %; urea, 4·5 %; uric acid, 68·5 %. The present authors therefore suggest that crocodiles should be regarded as *ammono-uricoteles*, since they excrete ammonia as the main nitrogenous end metabolite of the liquid urine, together with uric acid in the form of a white urinary deposit. Hopping (1923) and Coulson *et al.* (1950) in their studies on alligators made no mention of these deposits. Notwithstanding this, the present authors, from their previous experience with reptiles, assume that uric acid deposits are very probably present in alligators, since such deposits have been detected in many representatives of all reptilian orders. These deposits were detected in the order Chelonia represented by turtles (Khalil, 1947) and tortoises (Khalil & Haggag, 1955), in the order Lacertilia (Khalil, 1951 and Haggag, 1954) and in the order Ophidia (Khalil, 1948*a*, *b*). However, previous authors working on alligators may have overlooked the presence of such deposits which are usually found mixed with faeces, as alligators have no urinary bladders and no separate urinary apertures. If such an assumption is correct, alligators will be *ammono-uricoteles* like crocodiles and not ammonoteles as they have been considered until now according to the studies of Hopping (1923) and Coulson *et al.* (1950).

SUMMARY

1. In *Crocodylus niloticus* the liquid urine contains a white deposit. In the present study both the liquid and the deposit have been analysed.

2. For the liquid urine, the following values for percentage of total nitrogen have been found: ammonia, 66·8%; urea, 12·5%; uric acid, 2·3%.

3. For the deposit the corresponding figures are: ammonia, 6·0%; uric acid, 88·6%; urea absent.

4. From the relative proportions of liquid urine and deposit it is calculated that the total excretory nitrogen (liquid + solid) is distributed as follows: ammonia, 25·4%; urea, 4·5%; uric acid, 68·5%.

5. Crocodiles are thus ammono-uricotelic. The view that alligators are ammonotelic derives from analysis of liquid urine only. Uric acid deposits have been noted in the urine of reptiles of many orders and it is probable that they occur also in the urine of alligators.

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REFERENCES

Coulson, R. A., Hernandez, T. & Brazda, F. G. (1950). Biochemical studies on alligators. *Proc. exp. Biol. Med.* **73**, 203–6.

Haggag, G. (1954). Nonprotein nitrogenous excretion in some Egyptian reptiles. Unpublished Master's thesis, University of Cairo, Egypt.

Hopping, A. (1923). Seasonal changes in the gases and sugar of the blood and nitrogen distribution in the blood and urine of alligators. *Amer. J. Physiol.* **66**, 145–63.

Khalil, F. (1947). Excretion in reptiles. I. Nonprotein nitrogen constituents of the urine of the sea turtle *Chelone mydas* L. *J. Biol. Chem.* **171**, 611–16.

Khalil F. (1948*a*). Excretion in reptiles. II. Nitrogen constituents of the urinary concretions of the oviparous snake, *Zamenis diadema*, Schlegel. *J. Biol. Chem.* **172**, 101–3.

Khalil F. (1948*b*). Excretion in reptiles. III. Nitrogen constituents of urinary concretions of the viviparous snake *Eryx thebaicus*, Reuss. *J. Biol. Chem.* **172**, 105–6.

Khalil, F. (1951). Excretions in reptiles. IV. Nitrogenous constituents of the excreta of lizards. *J. Biol. Chem.* **189**, 443–5.

Khalil, F. & Haggag, G. (1955). Ureotelism and uricotelism in tortoises. *J. exp. Zool.* **130**, 423–32.

Lewis, H. B. (1918). Some analysis of the urine of reptiles. *Science*, **48**, 376.

Winterstein, H. (1924). Handbuch der vergleichenden Physiologie, p. 803. B.2, G. Fischer, Jena.

MEASUREMENT OF DISSOLVED OXYGEN IN SWAMP WATERS. FURTHER MODIFICATION OF THE WINKLER METHOD

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INTRODUCTION

There have been several modifications of the original Winkler method for the estimation of dissolved oxygen (Winkler, 1888) designed to overcome the sources of error due to the presence of oxidizing and reducing substances in natural waters and industrial wastes. In the course of work on tropical swamps we have been interested in the amount of oxygen in water which not only may contain large quantities of organic matter, ferrous or ferric iron, nitrites and other disturbing substances, but may also be deeply coloured or charged with sediment to give anything from a slightly turbid to a densely muddy liquid.

For such water none of the modifications so far described is satisfactory. It does not in fact appear that any independent check has been made on the absolute accuracy of any of these modifications, though the reliability of the original method has been proved by Wheatland & Smith (1955) by comparison with gasometric analysis of distilled water and sea water. An attempt is made to assess the absolute accuracy of the method described in this paper.

IODINE-DIFFERENCE METHOD

The various modifications designed to overcome the difficulties mentioned above have been reviewed by Ohle (1953). Since the Winkler method involves the final estimation of an equivalent amount of iodine, the most satisfactory as well as the most rapid is the 'sample-blank' or 'iodine-difference' method of which an improved version was developed by Ohle. This involves two original identical samples to each of which the same measured quantity of iodine is first added, and the alteration in the amount of free iodine by the disturbing substances is therefore the same in both. One sample (A) is then subjected to the Winkler procedure, and the same reagents are added in the wrong order to the other (B or 'blank'), which, unlike A, then contains no additional iodine equivalent to the dissolved oxygen. The difference between the titres (A–B) is equivalent to the amount of dissolved oxygen.

An earlier variant of this method involved adding the iodine to the alkaline iodide solution which, with manganous sulphate, comprises the Winkler reagents (Adams, Barnett & Keller, 1943). Ohle (1953) objects to this procedure because it is difficult to add exactly the same volume of an extremely alkaline solution to the two samples. The method described here avoids this difficulty in another way.

Ohle discovered an important source of error in dealing with what he called 'ein stark verschmutztes Wasser' from ponds. He found that the amount of free iodine left in the blank (B) became progressively less the longer the interval between adding the Winkler reagents and the final acidification. Since this did not apply to A, the apparent amount of oxygen became greater the longer this interval. This did not occur with clean water and he interpreted it as due to colloidal and particulate organic matter which was thrown down by the manganous hydroxide precipitate in A, but not in B where no precipitate is formed. He suggested that in the very alkaline conditions at this stage, when the added iodine is present as the very reactive hypoiodite, the suspended colloidal matter in B would be highly reducing and react with it. To obviate this error he advocated addition of acid immediately after the prescribed 10 sec. of shaking with the Winkler reagents. There was, however, no evidence that, during the minimum time needed to ensure completion of the Winkler reaction, there had not already been some reduction of iodine in B. This can only be decided by an independent estimate of the oxygen content, and it will be seen from the following that this error is not in fact overcome, even with moderately polluted water, by reducing the time taken over the operations to a minimum.

MODIFIED IODINE-DIFFERENCE METHOD

The procedure devised to eliminate the above error involves a preliminary clarification by aluminium hydroxide (Ellis, Westfall & Ellis 1948, p. 6) of a single sample of about 200 ml. from which the clear and decolorized supernatant is siphoned into two 35 ml. bottles, which are the A and B samples for the iodine-difference method. The added iodine is incorporated with the potassium hydroxide which produces the precipitate of aluminium hydroxide in the original sample, thus avoiding the difficult operation of adding exactly equal volumes of iodine solution to A and B. Clarification both removes colloidal organic matter and makes possible oxygen estimations in densely muddy water. A single original sample is preferable to two, because the identity of A and B in content of reducing substances is thereby assured.

The rest of the procedure is similar to that of Ohle (1953) except that, instead of altering the order of adding the reagents, saturated sodium chloride is substituted for manganous sulphate in the blank (B), and phosphoric acid is used in both because of the large amount of iron in swamp water.

The final titration with sodium thiosulphate was done with an Agla syringe micro-burette as in the micro-method of Fox & Wingfield (1938). Up to five 5 ml. aliquots can be taken from each sample.

REAGENTS

(1) Potassium aluminium sulphate. Saturated solution (about 11 %) in saturated NaCl.

(2) Iodine, 4 g.; KI, 100 g.; water, 100 ml.

(3) 19% KOH in saturated NaCl} equal parts by volume.
Reagent (2)

(4) $MnSO_4.4H_2O$, 50 g.; water to 100 ml.

(5) KI, 15 g.; KOH, 70 g.; water to 100 ml.

(6) NaCl saturated solution.

(7) Orthophosphoric acid.

(8) KIO_3 standard solution, e.g. 0·465 g./l.

(9) Standard iodine solution: (8) 2·00 ml., (7) 1 ml., KI crystals about 1 g., water to 100 ml.

(10) $Na_2S_2O_3.5H_2O$, 6 g.; NaOH 20%, 1 ml.; isobutyl alcohol, 10 ml.; water to 1 l.

(11) Sodium starch glycollate 5% in water (stock) diluted five times.

Reagents (1)–(6) are made up in freshly boiled distilled water to avoid supersaturation with air.

In (3) the iodine is in the form of hypoiodite leaving just sufficient free potassium hydroxide to precipitate aluminium hydroxide when added with (1) without raising the pH to a level likely to hasten the absorption of dissolved oxygen by the organic matter. It was shown in fact that the pH of a sample of swamp water, originally 6·5, was raised to 7·6 by this procedure, and that the copepod Crustacea in this sample remained alive above the precipitate for more than 5 hr.

PROCEDURE

The clarifying and Winkler reagents are injected with hypodermic syringes.

Operations (*a*)–(*d*) must be done in the field immediately after sampling.

(*a*) Samples are collected in rather tall 200 ml. reagent bottles. To each are added: reagent (1), 1·5 ml.; reagent (3), 0·6 ml.

The samples are stoppered, shaken vigorously and stood under water for the precipitate to settle. This takes 10–20 min. according to the nature of the suspended matter.

(*b*) With the simple device shown in Fig. 1 the clear supernatant is siphoned into the 35 ml. bottle A from which it passes into B. When B is full A has been sufficiently flushed and there is still enough water above the inlet of the siphon tube in the original sample to avoid contamination from the air above. This was confirmed by sampling deoxygenated water. A and B are stoppered and immediately treated with the Winkler reagents.

(*c*) To A are added: reagent (4), 0·17 ml.; reagent (5), 0·17 ml. The bottle is immediately stoppered and shaken for 10 sec. After standing until the precipitate has fallen from the neck of the bottle (10–20 sec.) 0·3 ml. of acid (7) is added and the bottle is restoppered and shaken until the precipitate is dissolved.

(*d*) To B are added: reagent (6), 0·17 ml.; reagent (5), 0·17 ml. This is then treated exactly as A, though no precipitate is formed.

A and B, after acidification, can be kept sealed and in the dark for at least 24 hr. without change in the iodine content.

(*e*) 5 ml. aliquots are titrated against thiosulphate (10) in an Agla syringe micro-burette after adding three drops of indicator (11). The thiosulphate is standardized against 5 ml. of the standard iodine solution (9).

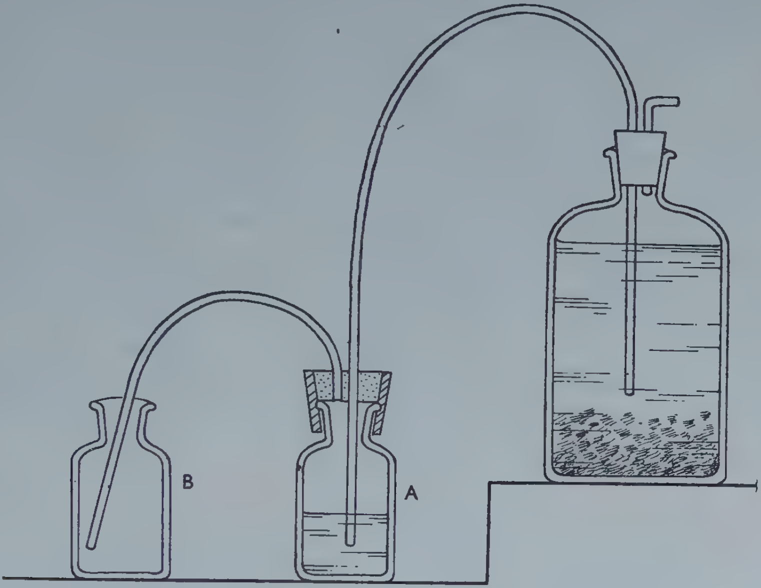


Fig. 1. The method for siphoning the A and B samples (each 35 ml.) from the supernatant of the original sample (200 ml.) after clarification. The double bung on bottle A ensures that this is flushed to the top so that it can be stoppered without including air bubbles. The Polythene tube passes loosely through the bung in the original sample bottle so that the depth can be adjusted according to the amount of precipitate.

CALCULATION

Oxygen in sample $= \frac{1}{5}KF(A-B)$ mg./l.

$K=$ factor converting micrometer units to mm.^{3}. This is approximately 20, but should be determined accurately by weighing mercury delivered from the burette over the range used.

$F=$ the oxygen equivalent of the standardized thiosulphate, in g. O_2/l.

A and $B=$ volume in micrometer units of thiosulphate equivalent to 5 ml. of A and B, respectively.

CORRECTION FOR OXYGEN IN REAGENTS

Some users of the Winkler method (Ohle, 1953) have made a correction for the oxygen added in the reagents, including the acid. It was, however, shown by Adams *et al.* (1943) that the oxygen in the acid does not react with the manganous hydroxide and that a correction for the oxygen in the Winkler reagents would amount to no more than 0·01 mg. O_2/l. A more direct proof of the insignificance of this correction was given by the comparison by Wheatland & Smith (1955) of gasometric with

Winkler estimations of dissolved oxygen in distilled water and sea water. The mean difference between the results from the two methods was 0·01 mg. O_2/l., the gasometric being the higher. No correction is therefore introduced here, but the clarifying reagents (1) and (3) are made up in a saturated solution of NaCl to reduce to the minimum the oxygen dissolved in them.

END-POINT OF IODOMETRIC TITRATION

An improved end-point was obtained by using a magnetic stirrer made of a piece of iron wire about 5 mm. long sealed in a glass tube. The rotation of this at the bottom of the titration tube, by a rotating magnet below, caused a vortex down the centre of the liquid. The tip of the microburette (hypodermic needle) was lowered by a screw-stand into the top of the vortex. On nearing the end-point the vortex could be distinguished as a column of fluid, clear compared with the surrounding faint bluish tint, just after a small amount of thiosulphate was injected. The end-point was reached when the injection of another division of the micrometer head (0·2 mm.^{3}) gave no colour differentiation of the vortex column. The titration tube was illuminated from behind through frosted glass. In the field stirring was done by mouth-blowing through a fine Polythene tube.

SPECTROPHOTOMETRIC ESTIMATION OF IODINE

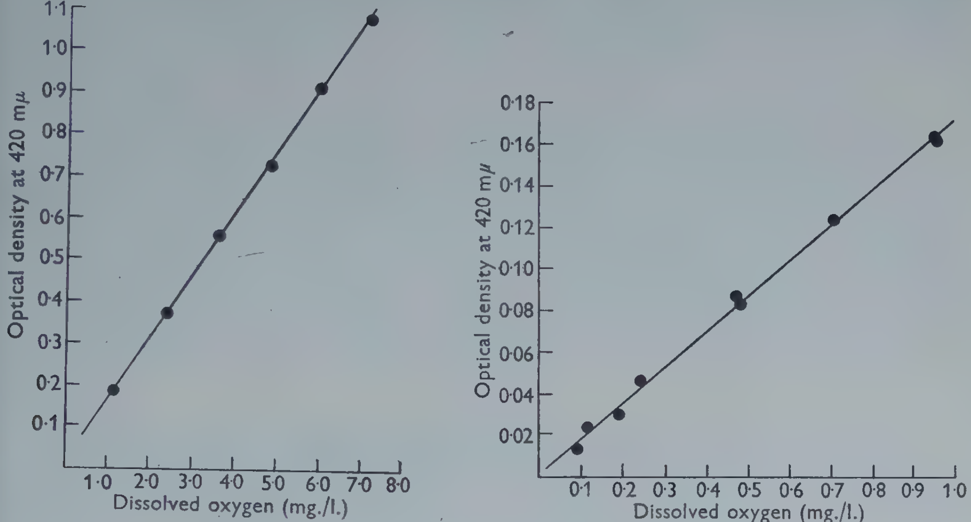
After the work described in this paper had been completed it was discovered that the final estimation of iodine could be done, as accurately as by titration and vastly more quickly, by direct measurement of optical density in a Unicam spectrophotometer (S.P. 600). Preliminary trials with a series of iodine solutions within the required range, in quartz optical cells of 1 cm. depth, showed that 420 mμ was a suitable wave-length, giving a steep curve of optical density against iodine concentration. The instrument was then calibrated by measuring the optical density at 420 mμ of a series of standard iodine solutions made up in water containing acidified Winkler reagents, the same reagents without free iodine being used as blank. Two curves were drawn (Fig. 2*a*, *b*) relating optical density to the oxygen equivalent of the iodine solution, one over the range 0–8 mg. O_2/l., the other on a larger scale over the range 0–1 mg. O_2/l., the latter covering most of the values normally obtained from swamp waters in the field. The relation is clearly linear.

Table 1 compares spectrophotometric with titrimetric estimations, each sample being finally estimated by both methods. It will be shown later that the present method, ending with titration, cannot be more accurate than ±0·2 mg. O_2/l. The agreement between the two is therefore satisfactory. The negative figures (i.e. $B > A$) obtained from stagnant swamp water are discussed below (p. 563).

The spectrophotometric estimation is particularly suited to the iodine-difference method because the sample containing the lesser amount of iodine (B) can be used as the blank and the difference is read directly.

To titrate in duplicate 5 ml. aliquots from one pair of A and B samples requires about 15 min. In 5 min. it is possible to estimate photometrically the difference in optical density between four pairs of samples and to read off the equivalent oxygen concentrations from the calibration curve.

Direct spectrophotometric estimation of iodine was used by Littmann & Benoliel (1953) for continuous recording of oxidants in the atmosphere, for which filters were used giving wave-lengths in the near ultra-violet (320–380 mμ). Ovenston & Watson (1954) used a Unicam spectrophotometer S.P. 500 to estimate small



(*a*) (*b*)

Fig. 2. Curves showing the relation between the concentration of free iodine (represented as the equivalent concentration of dissolved oxygen) and the optical density of the solution measured with a Unicam Spectrophotometer S.P. 600 at a wave-length of 420 mμ in quartz optical cells of 1 cm. depth, in the range (*a*) 0–8·0, and (*b*) 0–1·0 mg. O_2/l.

Table 1

Sample	Type of Winkler	Oxygen (mg./l.)		
		Spectro-photometer	Titrimetric	Difference
Boiled distilled water	Unmodified	1·02	1·03	−0·01
		1·85	1·88	−0·03
Tap water	Unmodified	7·20	7·11	+0·19
		2·22	2·09	+0·13
		6·78	6·82	−0·03
		4·81	4·64	+0·17
		6·90	6·74	+0·16
		1·58	1·59	−0·01
Stored swamp water	Present iodine difference	8·40	8·43	−0·03
		8·14	8·31	−0·17
		2·92	2·85	+0·07
		2·85	2·80	+0·05
Stagnant swamp water	Present iodine difference	−0·28	−0·20	−0·08
		−0·19	−0·13	−0·06
		−0·14	−0·01	−0·13
		−0·01	−0·08	−0·07

amounts of oxygen in boiler water by the Winkler method, the optical density of the iodine solution being estimated at 353 mμ. Briggs, Knowles & Scragg (1954) developed a continuous dissolved oxygen recorder in which iodine from the Winkler reaction was recorded spectrophotometrically at a wave-length of 430 mμ given by a filter. This is close to the wave-length used in the present method (420 mμ). They also found that the normal unavoidable variation in the amount of potassium iodide added during the Winkler procedure had no significant effect on the amount of light absorbed by the iodine solution.

ACCURACY OF MODIFIED IODINE-DIFFERENCE METHOD

In the highly polluted water of tropical swamps there is an unstable equilibrium between the reducing processes below and the penetration of oxygen from the air above. This results in a very steep oxygen gradient from the surface film downwards. A 200 ml. sample cannot therefore be drawn from a region of uniform oxygen content, and great sensitivity in the estimation would have no value. It is anyhow not to be expected, in view of the instability and of the great variety of reactive substances which could be present in such waters, that oxygen could be estimated with great accuracy.

Table 2

Swamp water previously aerated for 48 hr. at 30·0° C., and aeration continued throughout. A 200 ml. sample siphoned off daily together with a 35 ml. sample (figure in brackets) the latter analysed by the unmodified Winkler method. Barometric pressure 660 mm. mercury.

Days after start	Oxygen found (mg./l.)	Theoretical oxygen (mg./l.)	Difference (mg./l.)
2	6·50 (5·81)	6·50	0·0
3	6·40 (6·01)	6·50	−0·10
4	6·30 (6·05)	6·50	−0·20
5	6·43 (6·00)	6·50	−0·07
6	6·37 (6·00)	6·50	−0·13
7	6·35 (5·75)	6·50	−0·15

The accuracy of the method was tested on swamp water saturated with air (Table 2). About 3 l. of water were vigorously aerated through a diffuser for 48 hr. at constant temperature (30·0° C). During the next 6 days a 200 ml. sample was siphoned off daily and analysed. The theoretical amount of dissolved oxygen was calculated from the temperature and barometric pressure (660 mm. mercury) using the nomogram of Mortimer (1956). In addition a 35 ml. sample was taken daily and analysed by the unmodified Winkler method. The lower figures from the latter demonstrated the continued existence of reducing substances throughout the experiment.

The figures in Table 2 indicate that the sensitivity of the method for water of high oxygen content is about 0·2 mg. O_2/l.

It is obviously more difficult to assess the accuracy for natural waters containing very little or no oxygen, because they are likely to be in a very unstable condition and to contain much reducing matter. The problem was complicated by the discovery of 'negative' values with very polluted samples. Determinations on long-standing swamp pools, and on samples of swamp or pond water sealed in bottles for some days to exhaust the oxygen, gave a titre for B greater than that for A and thus a 'negative' oxygen figure equivalent to anything up to 0·5 mg. O_2/l. Whatever the cause of this phenomenon it was obviously necessary to be sure that 'negative' values are got only from samples containing no oxygen.

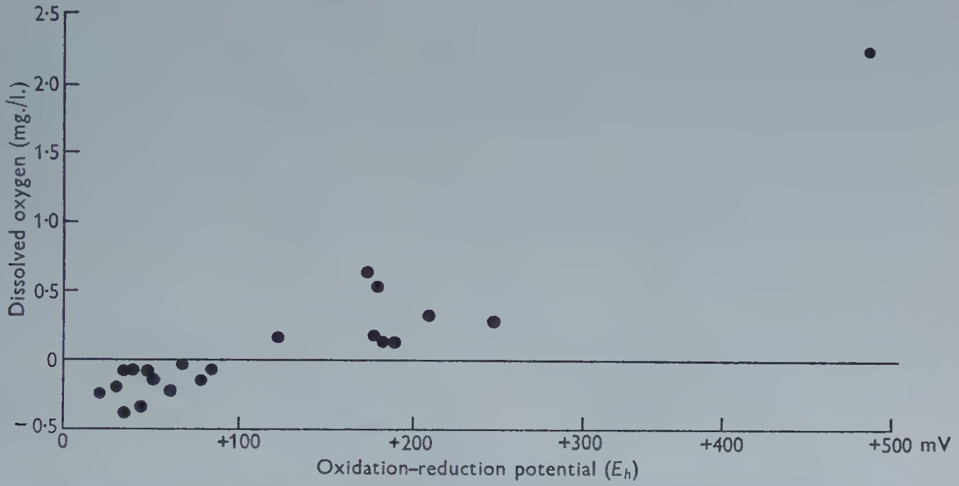


Fig. 3. The relation between estimated oxygen concentration and oxidation-reduction potential (E_h) of swamp-water samples, containing little or no dissolved oxygen. The negative oxygen figures denote that sample B contained more iodine than A. The pH of the samples was 6·6–6·8.

To decide this point a large tank was set up containing swamp water together with mud and decomposing vegetable matter. From this a pair of 200 ml. samples were siphoned in series to ensure complete uniformity. One was analysed by the present method, in the other oxidation-reduction potential was measured by a bright platinum electrode. This was lowered through the neck of the bottle to near the bottom, the sample being connected to the saturated calomel reference electrode by an agar bridge. With samples giving potentials (E_h) of less than +200 mV. the readings were reasonably steady after 5 min. Less reliance can be placed on the higher figures, but the latter are in fact of no interest in the present connexion. Readings were taken on a Cambridge potentiometer after 10 min. The pH of all samples was 6·6–6·8. Sampling was done over a period of a week. The water was siphoned from various depths and after varying degrees of stirring in order to get a wide range of oxygen concentrations.

The relation between oxygen concentration and E_h is shown in Fig. 3. Negative oxygen figures were obtained only from samples having E_h less than +100 mV. The work of Pearsall & Mortimer (1939) on oxidation-reduction potentials in natural waters and muds makes it clear that an E_h as low as $+100$ mV. can only be obtained in water devoid of dissolved oxygen.

Many attempts were made to discover the cause of this 'negative' phenomenon so that the procedure might be modified to avoid it. Complete failure in this, though unsatisfactory, does not appear in view of the above experiments (Fig. 3) to affect the reliability of the method. On the other hand, the 'positive' error in the standard iodine-difference method, discussed in the next section, is a serious disadvantage.

COMPARISON WITH STANDARD IODINE-DIFFERENCE METHOD

The preliminary clarification was introduced in order (*a*) to make possible estimations on very muddy waters, and (*b*) to avoid the error reported by Ohle (1953) in the standard iodine-difference method whereby, with polluted waters, the apparent oxygen-content increases with the time taken between adding the Winkler reagents and acidification, owing to progressive reduction of free iodine in B only. The experiments recorded in Table 3 show that the error cannot be avoided by reducing this time interval to the minimum possible.

Table 3

Samples	Oxygen (mg./l.)	
	Standard iodine-difference method	Present method
(*a*) From stagnant swamp pool, probably anaerobic. Sample pairs run off daily for 6 days	(i) 0·36	0·07
	(ii) 0·68	0·12
	(iii) 0·36	0·13
	(iv) 0·24	0·05
	(v) 0·86	Nil
	(vi) 0·80	Nil
(*b*) Hay infusion ($E_7 = -214$ mV.)	0·46	−0·11
(*c*) Filtered swamp water aerated for 1 hr.	(i) 5·08	4·25
	(ii) 4·97	4·18
(*d*) Pond water, stirred	4·32	3·88

In order to ensure that identical samples were taken for comparison of the two methods, the water was stored in an aspirator bottle and the two 200 ml. samples were run off simultaneously through a Y-tube. The standard method was followed by adding free iodine to the original sample, the requisite quantity being made up in a volume of saturated sodium chloride equal to the volume of the clarifying reagents used in the present method. The subsequent procedure was identical for both methods. The acid was added *immediately* after shaking for 10 sec. with the Winkler reagents.

In all cases the figures from the standard method were in excess of those obtained by the present method. It is significant that the lower the oxygen content the greater was the proportionate difference between these two figures. But it should be noted that there was a significant difference between the figures even when the water was not excessively polluted ((*c*) and (*d*)). We have reason to believe that figures obtained by the present method are correct to within 0·2 mg. O_2/l. (see previous section). In the case of the extremely polluted hay infusion in which there was undoubtedly no dissolved oxygen ($E_7 = -214$ mV.) and which was giving a negative figure by the present method (see previous section), the standard iodine-difference method gave 0·46 mg. O_2/l.

SUMMARY

1. A modification of the iodine-difference Winkler method is described which involves a preliminary clarification of a single sample, from which two smaller samples are siphoned.

2. This makes possible estimations on extremely muddy and polluted water and avoids an error in the standard iodine-difference method which is very large with highly polluted waters.

3. The iodine is added with the clarifying reagents to the original sample, thus avoiding the necessity for quantitative addition of iodine to two separate samples, which is another potential source of error in the standard method.

4. Estimations on swamp water saturated with air under standard conditions indicate that the method is accurate to within 0·2 mg. O_2/l.

5. With polluted water, having an oxidation-reduction potential (E_h) of less than +100 mV., 'negative' figures for oxygen content are obtained. The cause of this phenomenon could not be discovered but, since this potential indicates anaerobic conditions, the method is not thereby affected.

6. An improved iodine-titration end-point is described involving the use of a magnetic stirrer.

7. Direct spectrophotometric estimation of iodine is shown to be as accurate as, and very much more rapid than titration, and particularly suited to the iodine-difference method.

This work is part of a scheme of Research on Tropical Swamps financed by the Nuffield Foundation, and some of the apparatus used was bought with a grant from the Research Grants Committee of Makerere College. I am indebted to Mrs J. R. Nilsson for assistance during the later stages of the work.

REFERENCES

Adams, R. C., Barnett, R. E. & Keller, D. E. (1943). Field and laboratory determination of dissolved oxygen. *Amer. Soc. Test. Mat. Proc. 46th Meet.* **43**, 1240–57.

Briggs, R., Knowles, G. & Scragg, L. J. (1954). A continuous recorder for dissolved oxygen in water. *Analyst*, **79**, 744–52.

Ellis, M. M., Westfall, B. A. & Ellis, M. D. (1948). Determination of water quality. *Res. Rep. U.S. Fish. Serv.* no. 9.

Fox, H. M. & Wingfield, C. A. (1938). Determination of oxygen dissolved in a small quantity of water. *J. Exp. Biol.* **15**, 437–45.

Mortimer, C. H. (1956). The oxygen content of air-saturated freshwater and aids in calculating percentage saturation. *Mitt. int. Ver. Limnol.*, no. 6, 1–20.

Littmann, F. E. & Benoliel, R. W. (1953). Continuous oxidant recorder. *Analyt. Chem.* **25**, 1480–3.

OHLE, W. (1953). Die chemische und die elektrochemische Bestimmung des molekular gelösten Sauerstoffs der Binnengewässer. *Mitt. int. Ver. Limnol.*, no. 3, 1–44.

OVENSTON, T. C. J. & WATSON, J. H. E. (1954). The spectrophotometric determination of small amounts of oxygen in waters. *Analyst*, **79**, 383–7.

PEARSALL, W. H. & MORTIMER, C. H. (1939). Oxidation-reduction potentials in waterlogged soils, natural waters and muds. *J. Ecol.* **27**, 483–501.

WHEATLAND, A. B. & SMITH, L. J. (1955). Gasometric determination of dissolved oxygen in pure and saline water as a check of titrimetric methods. *J. appl. Chem.* **5**, 144–8.

WINKLER, L. W. (1888). Die Bestimmung des in Wasser gelösten Sauerstoffs. *Ber. dtsch. chem. Ges.* **21**, 2843–54.

THE CO-ORDINATION OF INSECT MOVEMENTS

III. SWIMMING IN *DYTISCUS*, *HYDROPHILUS*, AND A DRAGONFLY NYMPH

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Although many of the structural adaptations associated with the locomotion of aquatic insects are well known, there are few observations on the details of their propulsive mechanisms. In general, these may be subdivided into those which effect swimming by undulatory movements of the body (Kalmus, 1936; Gray, 1953), by oar-like movements of the legs or wings (e.g. *Polynema*, Hymenoptera), or by jet propulsion methods involving either the use of a surface active secretion (*Stenus*, Staphylinidae) or the active expulsion of water from the insect. Of these the use of legs as oars is the most common among adults. In this paper cinematographic analyses are given of two beetles which use this method and of the abdominal and leg movements involved in the jet propulsion of a dragonfly nymph. In addition, some observations are described on the effect of amputating legs on the swimming of *Dytiscus*. These differ significantly from the observations of Bethe & Woitas (1930) and support conclusions based on a study of cockroach movements (Hughes, 1957).

MATERIALS AND METHODS

The species used were the giant water-beetles, *Dytiscus marginalis* and *Hydrophilus piceus*, and late instar nymphs of *Anax imperator*. Swimming of the beetles was observed in a large sink containing water about 6 in. deep. Films were taken while the insects swam freely above a glass plate marked with a grid and placed in the field of the camera. Similar films were taken of the dragonfly nymphs but the results were not satisfactory for all purposes. Close-up films, either in side or in dorsal view, of nymphs freely suspended from the wing pads proved of much greater value in revealing the sequence of events in the swimming cycle. Spots of enamel paint placed on the abdominal segments and legs helped in plotting their position on successive frames. Sinclair 35 mm. and Zeiss Movikon 16 mm. cameras were used at speeds of 24–30 frames/sec. for the beetles, but much faster speeds (up to 60 frames/sec.) and briefer exposures were necessary to 'fix' the dragonfly movements. General lighting was provided by two Photofloods and in addition carefully placed Point-o-Lites were used for the close-up shots.

Additional information about the events during swimming of *Anax* was obtained from films and simultaneous recordings on an oscilloscope of the time course of pressure changes within the branchial chamber and/or the forces on the animal resulting from the jet. As these events are extremely rapid great care was taken in the selection of recording apparatus and in the method of synchronizing films and oscillograph records. The wing pads of the insect were attached by a small paper clip to a short arm soldered to the anode of an RCA 5734 mechanotransducer valve. Although the natural frequency of the latter is 12 kcyc./sec. it was reduced considerably by these attachments. After suitable reductions in the length of the arm it was raised to 120 cyc./sec. which is sufficiently high to reproduce faithfully the true time course of the forces generated by the jet. Similarly, by using a Hansen condenser manometer (Hansen, 1949) with a natural frequency of nearly 100 cyc./sec., the time course of the pressure changes was accurately recorded even when fine (0·5 mm. outside diameter) hypodermic tubing was used. The following method of synchronization was suggested by Dr R. H. J. Brown, to whom I am grateful for advice on this and on other matters of technique. The point where a wire on a time marker, rotating $3\frac{1}{3}$ times/sec., touched a mercury drop was brought into focus in the camera field by a lens or mirror; this contact completed a circuit which modulated the intensity of the beams on the oscilloscope. It was thus possible not only to see which frame was related to the signal on the oscilloscope trace, but also to decide whether the frame was a fraction of a second before or after the signal by observing the actual position of the rotating contact on that frame.

RESULTS

(A) *The swimming of* Dytiscus *and* Hydrophilus

(1) *Morphological*

Dytiscus and *Hydrophilus* show many structural adaptations to their aquatic habitat some of which display striking convergences. On the whole, *Dytiscus* is better adapted to aquatic conditions than *Hydrophilus* which is correspondingly more efficient on land. The body is keel-shaped in both genera and offers little resistance to water flow, but it is in the legs that the most striking adaptations are found. The femur, tibia and tarsus of the meso- and metathoracic legs are all flattened. The hindlegs of swimming beetles are shorter relative to the body length than in terrestrial forms and the proportions of the segments are different. In Dytiscidae the tarsus is about twice the length of the tibia, whereas in terrestrial beetles they are nearly equal (Roth, 1909). Hairs are found on the tarsal joints of *Hydrophilus* and on both the tibia and tarsus of *Dytiscus*. In both genera these tarsal joints overlap one another on the surface which is anterior during retraction and thus increase the rigidity of the structure. They have a mechanism whereby the tarsi of the middle and hind pairs of legs can rotate, enabling them to 'feather' during the forward stroke. In *Dytiscus*, the articulation of the proximal segment with the tibia is similar to a ball-and-socket and facilitates a 100° rotation of the tarsus. This joint is fairly normal in *Hydrophilus*, but the articulation between the two proximal joints of the tarsus is very oblique and the rotation principally takes place about its axis. The other segments of the leg are also modified in both genera. Promotor-remotor movements of the coxae are completely excluded in *Dytiscus* as the coxae are fused to the sternum, while in *Hydrophilus* they are restricted by the coxae being sunk into spaces in the sternum. This adaptation probably serves to lessen the drag of the insects in the water.

The coxo-trochanteral joints are dicondylic and determine the movement of the femur very rigidly. This movement is not in one plane, because of the shape of the trochanter, and has the result that the tip of the femur moves parallel to the ventral body surface throughout its stroke. The femur is flattened and curved so that it is almost flush with this surface throughout the arc of its movement, adaptations which will again decrease the resistance to forward movement.

(2) *Cycle of hindleg movements*

A series of diagrams (Fig. 1) showing the position of the hindlegs throughout the cycle has been constructed from the photographs of the swimming insect. At the completion of the effective stroke (ii), the hindleg of *Dytiscus* appears narrow in the photographs because the flattened surfaces of the tibia (*a*) and tarsus (*b*) are pressing against the water and the hairs are spread out dorsally and ventrally to offer their maximum resistance. Protraction commences with flexion of the coxotrochanteral and femoro-tibial joints (iii). The tarsus rotates 100° (*b* to *d*) so that the edge which was anterior during retraction now becomes dorsal and the outline becomes blurred as the hairs are visible (iv). Roth (1909) described this tarsal rotation but

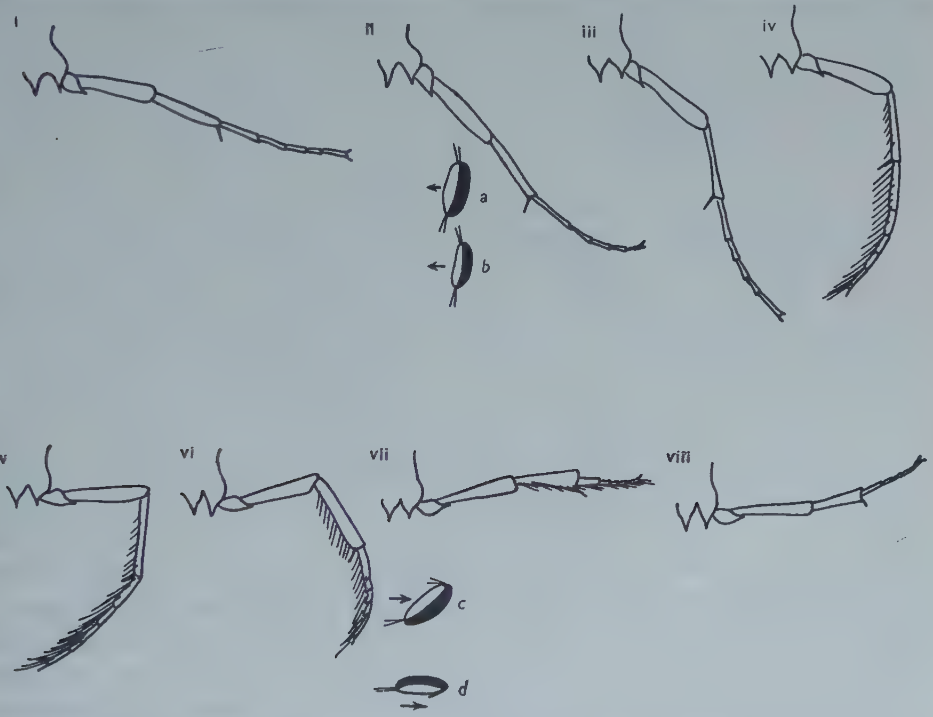

Fig. 1. *Dytiscus*. Diagrams (i)–(viii) show successive positions of the right hindleg during a typical cycle of swimming movements as seen in *dorsal* view. The rotation of the tibia (*a* and *c*) and tarsus (*b* and *d*) are indicated by diagrams of their cross-section at phases (ii) and (vi) of the cycle.

in the opposite direction. His account was shown to be wrong by filming insects with the tarsi painted white on one surface only. The anterior side during retraction becomes dorsal as the leg is drawn forwards and paint on its surface is only visible during protraction. The femur continues to protract and it forms the leading edge of the limb behind which trail the flexed tibia and tarsus (v). The tibia now begins to extend and takes up its fully protracted position (vi, vii). As Amans (1888) described, the rotation of the tibia (*a* to *c*) which occurs during the cycle is about 45°, but in the opposite direction to that of the tarsus. The femur is beginning to retract at this stage and as the tarsus rotates its tip passes dorsally to the rest of the limb. As the femur has already retracted by the time the tarsus is presenting its maximum surface to the water, it is probable that its extension and rotation are entirely passive. This may be partly true of the femoro-tibial joint also. The musculature agrees with this suggestion for, as Bauer (1910) pointed out, the extensor tibiae is a much smaller muscle than its antagonist and there are no extensors of the tarsus. During retraction the tibia rotates progressively until it is inclined as shown in (*a*) at the end of the active stroke. The whole limb acts as an almost straight oar (i) and the tarsi are curved by the water pressure. The films of insects with painted tarsi showed that during retraction the posterior surface of the tarsus is inclined slightly upwards which will produce a force tending to move the insect downwards. Towards the end of the stroke, the tibia flexes while the tarsus is still exerting pressure (iii) and protraction begins once more.

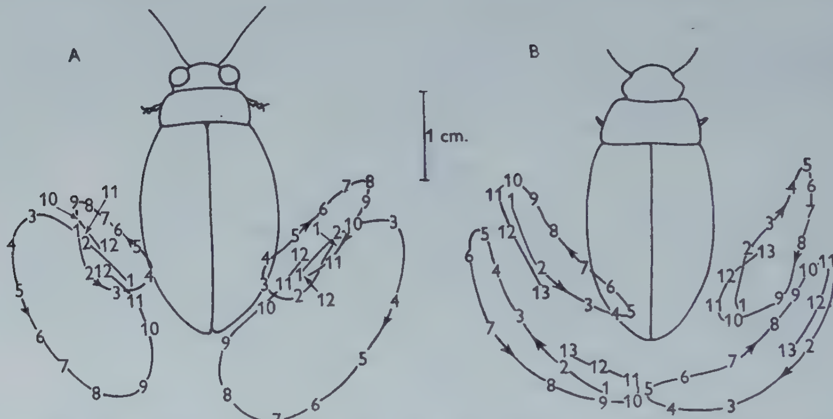

Fig. 2. Plots of the position of the tips of the hind- and middle legs on successive frames of a ciné film (30 frames/sec.) during normal swimming of *Dytiscus* (A) and *Hydrophilus* (B). In both cases the insect was turning slightly to the left.

This description indicates the principle underlying the 'rowing' movements of aquatic insects. It is highly developed in *Dytiscus* and the individual movements of the swimming legs of *Hydrophilus* conform to the same general plan. A comparison of the path followed by these legs illustrates the greater efficiency of *Dytiscus* in its swimming movements (Fig. 2A). During protraction of the legs in *Hydrophilus* (Fig. 2B) there is relatively little flexion of the femoro-tibial joint and the course followed by the limb tip during protraction is consequently further away from the body and offers a greater resistance to forward movement of the body. On the other hand, the middle legs of *Hydrophilus* exert a more powerful thrust against the water than does this pair of legs in *Dytiscus*. In both insects the highly-developed extensor trochanteris is the most important muscle in propelling the body forwards.

These movements of the legs during the swimming of *Dytiscus* are obviously quite different from those employed by the insect when walking, but in *Hydrophilus* the differences are not so well marked. The greater activity of the flexor tibiae muscles and the increased promotor-remotor movements of the coxae, result in the path followed by the hindlegs being much closer to the body when the insect walks.

(3) *The rhythm of the swimming movements*

In *Dytiscus*, the rhythm in which the legs are retracted is completely different from that described for the walking insect (Hughes, 1952). The two legs of a limb pair retract simultaneously, ensuring that the body is subjected to only very slight turning moments (Fig. 3A). When swimming along a path which is not perfectly straight the timing of a pair of legs may be altered. Fig. 2A was plotted from such an insect and it will be noticed that the right legs are retracted a little in advance of the left legs and also with a greater amplitude. Both these features will tend to correct the slight turn to the right which was made in the previous stroke. More rapid turning takes place when one of the hindlegs is held outstretched in the protracted position, while the contralateral leg retracts actively (Fig. 4B). The extended hindleg and also the middle leg on that side may make slight 'back-paddling' movements. The duration of protraction and retraction are usually equal in this insect, but sometimes protraction is shorter as in Fig. 4A. The difference is slightly exaggerated here, however, because the legs appear to be in a protracted position for a longer period on account of the inclination of the body. Previous accounts of the rhythm hardly mention the middle pair of legs and suggest that they perform only occasional movements during turning and to preserve the balance. This is true when the insect is undisturbed and swims slowly but in more rapid swimming, these legs play a regular part and retract together but in opposite phase to the hind pair of legs (Figs. 3A, 4). One function of the middle pair is to stabilize the body during the recovery stroke of the hindlegs. Their retractor movements are directed more ventrally than those of the hind pair, and this tends to raise slightly the front part of the body. For instance, in Fig. 4A the apparent changes in length of the body are plotted beneath the leg movements. A decrease in length is due to the abdomen being raised relative to the head by the buoyancy of the air store. As the middle legs retract, the body becomes more level in the water and its apparent length increases. It is noticeable that the length starts to decrease before the hindlegs are completely retracted, suggesting that perhaps they do not play such a great part in regulating the horizontal position of the body. The middle legs also function to maintain a uniform speed of forward movement, since when they are amputated progression is somewhat jerky, especially when the insect is excited and the body does not keep on too even a keel.

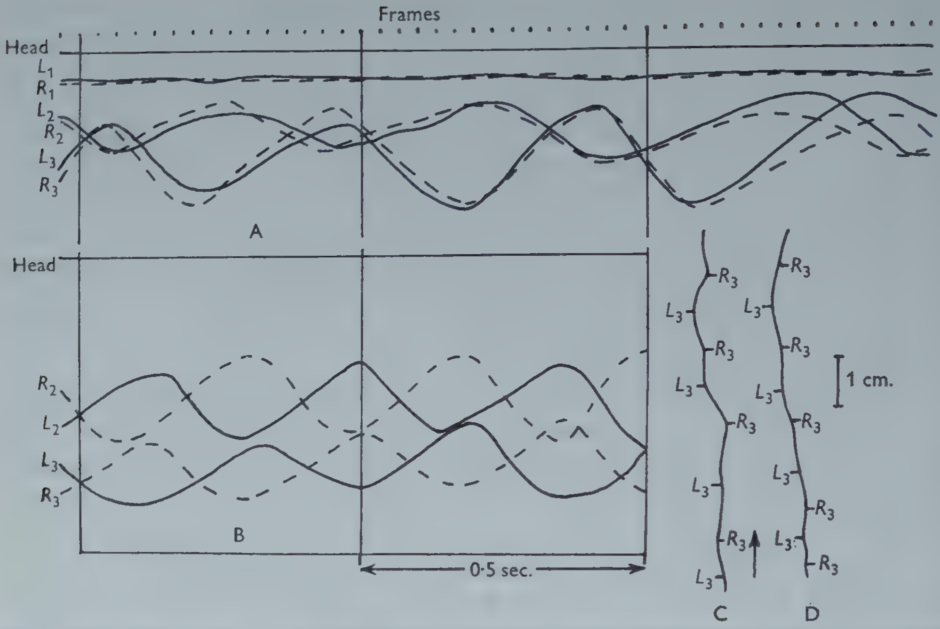

Fig. 3. The rhythm of leg movements during swimming plotted relative to the head in A *Dytiscus* and B *Hydrophilus*. C. The path of the head relative to the ground during B is shown, together with the instant when the hindlegs begin to retract. D. The same insect swimming along a straighter path.

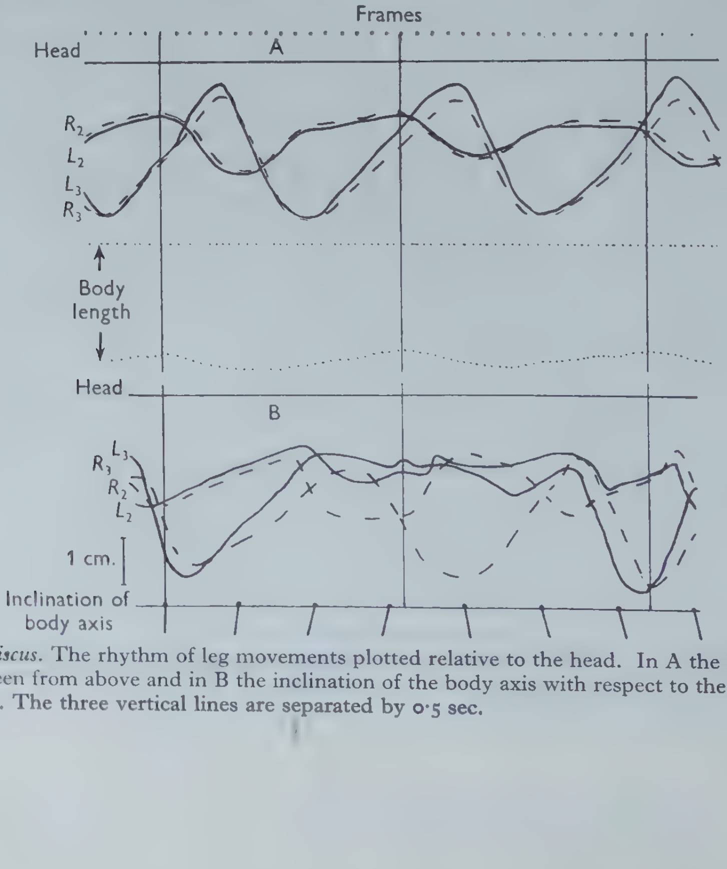

Fig. 4. *Dytiscus*. The rhythm of leg movements plotted relative to the head. In A the length of the body seen from above and in B the inclination of the body axis with respect to the grid are also plotted. The three vertical lines are separated by 0·5 sec.

The rhythm of leg movements in *Hydrophilus* differs from that of the Dytiscidae in that the two legs of a segment are in opposite phase. The middle and hind pairs are both employed and diagonal legs retract at exactly the same instant (Fig. 3B). The fully retracted middle legs lie beneath the body so that it is difficult to determine whether this is also true at the beginning of protraction. The duration of protraction of the hindlegs tends to be longer than retraction by about a fifth of a cycle. The position of the head of this insect has also been plotted in Fig. 3C, D and demonstrates that the path followed by *Hydrophilus* is much straighter than has been generally supposed. Deviations of the head to the right and left are associated with retraction of the hindleg on the opposite side. One of the reasons why these deviations are not so great is because the legs move simultaneously on the two sides. Synchronous retraction of diagonal legs is of further significance in that it distinguishes the swimming from the walking rhythm where there is always a slight delay between the instant at which R_2 and L_3 or L_2 and R_3 are retracted (Hughes, 1952).

(4) *The effects of limb removal in* Dytiscus

Bethe & Woitas (1930) described changes in the swimming movements which occur when one or more legs are amputated. The most striking changes take place when a single hindleg is removed. In such an insect they describe how the middle leg on the same side, previously inactive, became important in swimming and retracted simultaneously with the intact hindleg. However, analysis of films (Figs. 5, 6) taken in the present investigations has failed to confirm this and other details of their description. It can be readily observed that when the left hindleg (L_3) is amputated, the suddenly alarmed insect tends to circle to the left owing to the action of the right hindleg, but it soon swims along a straighter path because of the following modifications in leg movements:

(i) The intact hindleg (R_3) does not retract so far back as in the normal insect and during protraction it is not feathered so closely to the body nor does it move so far forwards. The movements of this leg do not have such a regular rhythm and give the impression of being constantly regulated. All these modifications tend to decrease the tendency for this leg to rotate the insect anticlockwise.

(ii) The left middle leg (L_2) continues in the normal rhythm so that it retracts as soon as R_3 has completed its effective stroke. The amplitude of its movements is increased both anteriorly and posteriorly.

(iii) Retraction is delayed in R_2 which therefore remains outstretched while L_2 retracts and, together with the protracting hindleg, acts as a fulcrum about which the body pivots.

Modifications (ii) and (iii) both increase the turning effect of L_2 and it has been noted already that similar changes occur in the normal insect when it turns (Figs. 2A, 4B). The net effect of the changes in rhythm and posture of the legs is that progression towards a given point is accompanied by oscillations which are due to an alternation of the turning moments produced by R_3 and L_2. This is clearly shown by tracing the path of the head or centre of gravity (Fig. 5). The overall path is

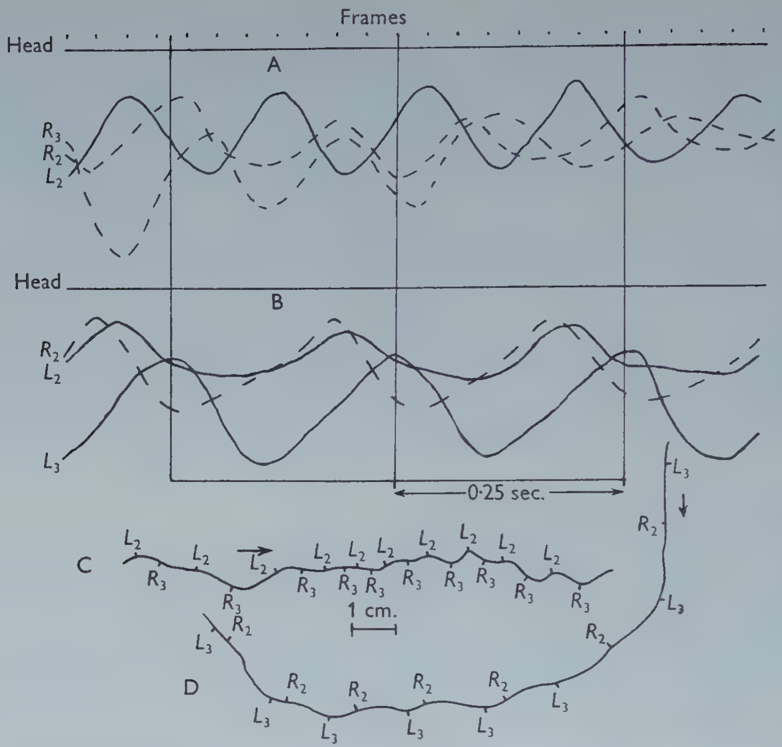

Fig. 5. *Dytiscus*. The rhythm of leg movements following the amputation of a single hindleg plotted relative to the head. In A the left hindleg (L_3) has been removed and the path of the head is shown in C. In B the right hindleg (R_3) was amputated and the insect was also blinded on the left side. This insect tends to circle more as is shown in D. In C and D the instants when the legs begin to retract are indicated.

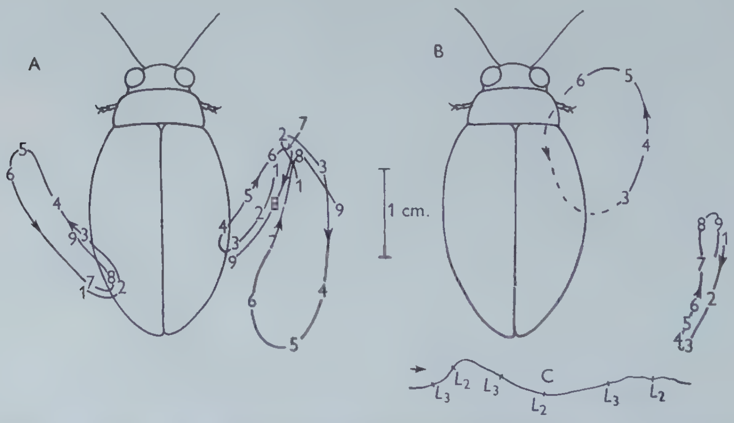

Fig. 6. *Dytiscus*. The position of the limb tips relative to the body on successive frames of ciné films (30 frames/sec.). The left hindleg has been removed in A which is the same insect shown in Fig. 5 A, C. In B both the middle and hindleg on the left side have been removed. Its path through the water and the instants at which L_3 retracts and L_2 protracts are shown in C.

fairly straight, which would not be the case if these modifications had not taken place. Bethe & Woitas (1930) did not plot the movements of the legs from their photographs, which in fact clearly show that the intact hindleg and contralateral middle leg do *not* retract simultaneously. Most later illustrations of this phenomenon (e.g. Wigglesworth, 1953) reproduce Bethe's diagram and not the actual photographs.

Observations on the effects of amputating other legs differed slightly from those of Bethe & Woitas but will not be described here. Changes in posture of the remaining legs occur in addition to their taking over of the swimming function. One very striking example of this occurs if both the hind- and middle legs of one side (L_2 and L_3) are removed. As was to be expected the insect tended to move to the left, again especially when it was startled. But it was able to swim along a relatively straight path (Fig. 6C) surprisingly well when left undisturbed. In doing this the intact middle and hindleg both altered their normal movements a great deal although the rhythm remained relatively unaltered. The hindleg was held outstretched and scarcely feathered during protraction, which was longer than retraction. The middle leg, however, protracted very much farther forwards and during this phase was held away from the body. During retraction it moved ventrally and its active stroke was directed under the body and towards the left-hand side. This leg, therefore, moved in an anticlockwise direction (Fig. 6B) and tended to rotate the head towards the right. Progression was inevitably slow by this method which reminds one of the manoeuvres which can be made with a canoe paddle.

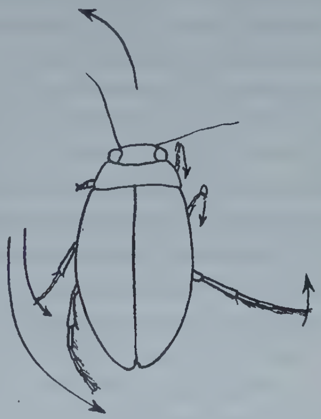

Fig. 7. *Dytiscus*. Diagram to illustrate the pattern of leg movements during the compensatory response produced when the insect is rotated in an anticlockwise direction.

Several neurological mechanisms are involved in the production of these plasticity changes and while a complete analysis cannot be given some responses of intact *Dytiscus* are suggestive. It has already been noted how during a slightly 'wavy' course and when turning (Figs. 2A, 4B) the posture and rhythm of the legs are modified which is accentuated following amputation. Even more striking is the observation that when *Dytiscus* is held and moved through water in a circle of about 6 in. diameter, a characteristic compensatory response is invariably observed (Fig. 7). The outer hindleg is outstretched and shows little active retraction, but occasionally moves forwards in this stiff condition in a way very reminiscent of the single hind-leg of the insects described above. At the same time, the other hindleg retracts strongly and frequently, while the two middle legs show movements which would also produce rotation in the opposite direction to the imposed movement. The most important sensory structures involved in this response are the eyes, antennae, and receptors on the legs themselves. The response persists in red light, but is not so marked after covering the eyes with wax containing lampblack, or when a striped environment is rotated at the same speed as the insect. If the antennae are also removed the response is sometimes absent and if it occurs is not very definite. During forced rotation of an insect there will, of course, be an asymmetric stimulation of the legs on the two sides because of the centrifugal force as is shown by the positions assumed by the legs when a dead insect is rotated.

It has not been possible to prove conclusively that any one of these receptors is solely responsible for this response. It seems likely that all three are able to produce it by themselves. The eyes are certainly involved a great deal in the orientation of this insect (Zeiser, 1934). They are probably the most important receptors as was concluded by Tonner (1938) in his study of similar compensatory responses of dragonfly nymphs. The importance of differential stimulation of antennae has not been discussed previously in *Dytiscus* but it must certainly occur even in normal swimming. The antennae are held forwards at an angle to the axis of progression and when the path deviates slightly a difference in their degree of bending is apparent on the films. The basal joints are profusely supplied with sensory structures which include Johnston's organ, a row of campaniform sensilla and several hair plates at the articulation. Both slowly and rapidly adapting responses to deflections of the antennae have been recorded from the antennal nerve. If a fine jet of water is directed at the antennae of a fixed *Dytiscus* from in front and to one side the contralateral hindleg usually kicks.

The above observations suggest that if the insect departs from a straight course the central nervous system will receive a changed pattern of sensory impulses from these sources and that the normal responses tend to restore the original orientation. As these responses have much in common with the alterations in motor activity which follow limb amputation, it is probable that these too are responses to the inevitable changes in peripheral inflow resulting from the tendency to circle. If insects with a single hindleg amputated are moved through the water in a straight path the remaining leg movements are almost normal. Correspondingly, the alterations in movements do not occur if the hindleg is removed from a blinded insect so that the insect circles for some time, particularly if the antennae are also removed. Insects which show almost rectilinear swimming after a hindleg has been amputated immediately circle when blinded. Some compensation does eventually take place in these insects, however, because of the proprioceptive inflow from the legs, but the majority of their movements are normal. Unilaterally blinded insects with a hindleg removed tend to circle, but may move fairly straight for short distances (Fig. 5 D). In this insect the intact hindleg moves normally and the two middle legs retract almost simultaneously (Fig. 5 B).

The supra- and suboesophageal ganglia must play a large part in the integration of the inputs from these different sources. Several lines of evidence suggest that they probably contain a mechanism which compares the inputs from the two sides of the body (cf. Kalmus, 1949) and controls the responses which occur when the insect departs from a given path. The latter need not necessarily be directly to or away from a given stimulus but can vary in its orientation. The factors controlling the 'set' of this mechanism may involve central as well as peripheral patterns of stimulation, but once the 'set' has been established changes in peripheral inflow will be analysed with respect to it. Such a mechanism is essential otherwise the compensatory responses would prevent the insect from turning. Unilateral blinding or extirpation of a supraoesophageal ganglion so alters the balance of the co-ordinating mechanism that persistent circling results.

Faivre's (1857) observation that the direction of circling is away from the injured side has been substantiated by several workers and Baldus (1927) has established that alterations in the movements of the legs on both sides of the body are involved. His photographs show that they are strikingly similar to those reported above following unilateral amputation of legs or rotation of the insect. Circling away from the blinded or decerebrate side continued even when the middle and hindlegs were amputated on one side. If the legs removed were from the side opposite to that from which the supraoesophageal ganglion was extirpated, circling resulted from only slightly exaggerated movements of the remaining middle and hindlegs. If, however, the amputation and brain injury were on the same side the leg movements were very different. His description is almost identical to that given above when the two legs were amputated but there was no brain injury. Baldus mentions that he observed this activity but he holds quite the opposite view to Bethe & Woitas, i.e. that following limb amputation the remaining legs move quite normally and that these asymmetries occur only after unilateral brain injuries have also been performed.

It is evident that the brain has a large number of both homo-lateral and contralateral connexions with the lower motor centres, but their complexity makes any description at this stage not only inadequate but probably misleading. Nevertheless, the very striking similarities between the leg movements following the amputation of a single hindleg and those involved in the compensatory and circus movements strongly suggests the operation of a single co-ordination mechanism influenced by peripheral changes and is quite contrary to the plasticity theory of Bethe.

(B) *The swimming of the dragonfly nymph*, Anax imperator

Normally these predators creep extremely slowly among weeds and await their prey, but they can swim quite rapidly especially when disturbed. They usually swim when placed in a large volume of water particularly if there are few objects to which they can attach themselves. Nymphs also swim if the legs are free when they are suspended from their wing pads by a small paper clip fixed to a steel rod. Periods of swimming are often produced when the rod is struck or the insect touched with a brush.

Jet propulsion is achieved by an extremely rapid contraction of the abdominal musculature (Whedon, 1918; Tonner, 1936; Snodgrass, 1954) producing a pressure head within the enlarged hindgut which forms the branchial chamber. Water escapes as a fine jet at high velocity from the anal opening which is guarded by valves. The reaction from this jet propels the insect forwards at speeds which reach 30–50 cm./sec. during the first centimetre of propulsion. The frequency of the jets may be up to

3/sec. and they may continue for some time but in a fixed insect a pause usually occurs after ten to twenty of them. These features were conveniently recorded when the insect was fixed to the RCA 5734 transducer valve. The time course of the propulsive forces generated by the jet was also registered and as Figs. 9 and 11 show, a maximum value of 0·6 g. is reached within 0·03 sec. and the total duration is a little under 0·2 sec. The area beneath the curve is a measure of the impulse acting on the insect and is equal to the change of momentum which was about 52 dyne-sec. in an actual experiment. As the insect weighed 1 g. the expected velocity had the insect been free was 52 cm./sec. This figure, however, neglects the drag forces to which the free insect would be subjected, but, even so, it is in good agreement with speeds measured from films at the onset of swimming.

The precise sequence of muscular movements is difficult to establish as the total duration of the contraction is only about 0·1 sec. The most obvious movements are the longitudinal and dorso-ventral contractions of the abdomen, and the retraction of all six legs so that they lie alongside the body. When the insect is held stationary the legs come forwards again before the next jet (Figs. 8–10), but this does not occur normally because of an 'Angelegtbleiben' reflex (Tonner, 1935) elicited by water stimulating the antennae during motion. The two legs of a segment usually retract simultaneously, though cases have been observed where only the three legs on one side retracted. Some shots suggest that the order of retraction is hind, middle, fore, but the intervals separating these movements are extremely small, being at most 1/50 sec. (Fig. 8). One of the difficulties is to decide the exact phase at which retraction of a leg is associated with a given jet, but usually this is recognizable when a leg moves more rapidly backwards, regardless of its other activity.

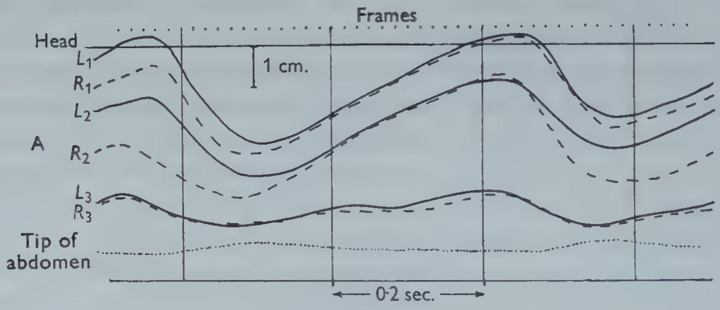

Fig. 8. *Anax*. Movements of the legs and tip of the abdomen relative to the head plotted from a film of the stationary insect.

The interval between the abdominal and leg movements is brief and in many cases they appear to be simultaneous. Most analyses suggest, however, that the hindlegs, at least, retract slightly before the abdomen shortens. Transducer records taken simultaneously tend to confirm this as there is a slight forward force synchronous with the leg movements and slightly preceding a rapid rise which coincides with shortening of the abdomen as the jet efflux occurs. The resulting propulsive forces on the insect cease when the abdomen is maximally shortened (Fig. 9). Flattening of the sternum seems to occur a little before the abdomen shortens, but it does not reach a maximum until after the abdomen has partly extended again (Figs. 9, 10). Water probably begins to be drawn in when the abdomen lengthens and continues to do so as the sternum is depressed. The most striking feature of the whole response is the longitudinal contraction, however, which may cause the abdomen to shorten by 7–10% of its resting length. Most of this appears to take place in the posterior segments but plots of the positions of individual segments (Fig. 10) shows that all segments are involved to some extent. The largest

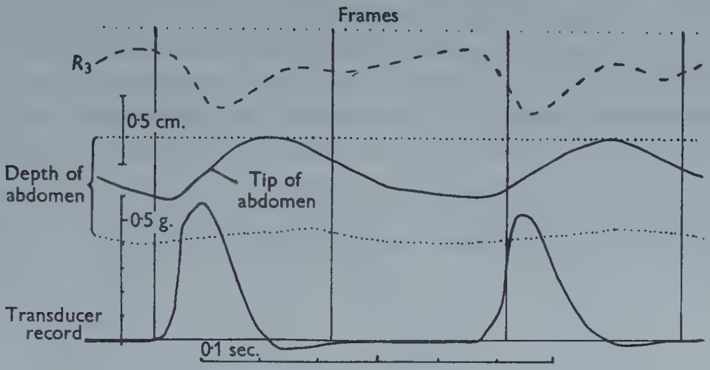

Fig. 9. *Anax.* The movements of the right hindleg and the tip of the abdomen plotted relative to the head. The depth of the 6th abdominal segment as seen in side view is also shown. A transducer record of the forces acting on the stationary insect as a result of these movements has been superimposed.

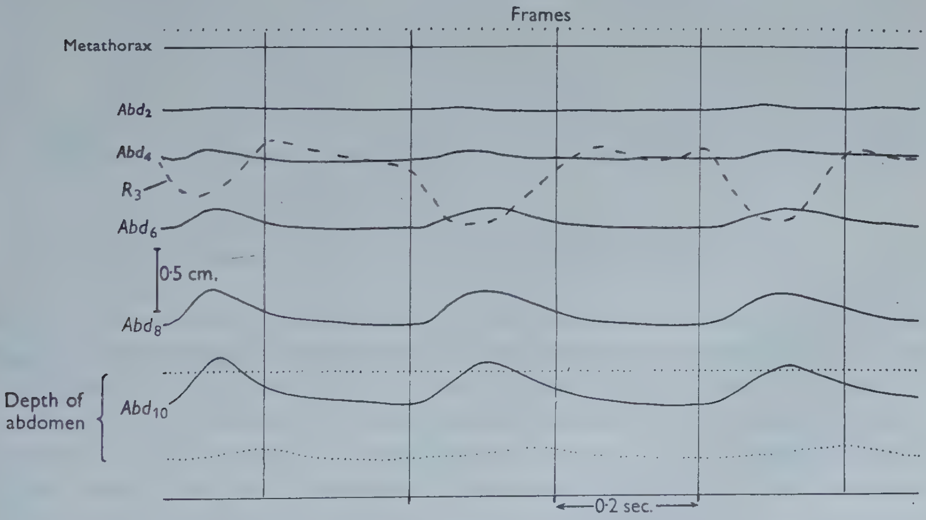

Fig. 10. *Anax.* The movements of different segments of the abdomen are plotted relative to the metathorax during swimming movements of a fixed insect. Movements of the right hindleg relative to the head and of the dorsal-ventral flattening movements of segment 6 are also shown.

proportion (40%) occurs in segments 6–8 and this is also the region where flattening of the sternum is greatest. These observations correlate with the internal anatomy as the branchial chamber is situated in these segments which are therefore the region where the maximum decrease of volume would be expected when water is expelled. The segments do not all contract at exactly the same instant as can be seen particularly during some of the slower contractions. The hind segments contract first and the telescoping spreads forwards very rapidly. The very first part of the whole response appears to be the closing together of the three valves which guard the external opening. It has not been possible to obtain good films of the valves, but direct observation certainly suggests that the aperture is completely closed just before the longitudinal contraction commences. It then appears to open slightly as water is expelled and later suddenly opens very widely and water is drawn in. The phase of valve closure is often associated with the slight apposition of the epiproct and two paraprocts. Opposite each of these terminal appendages is situated one of the three anal valves.

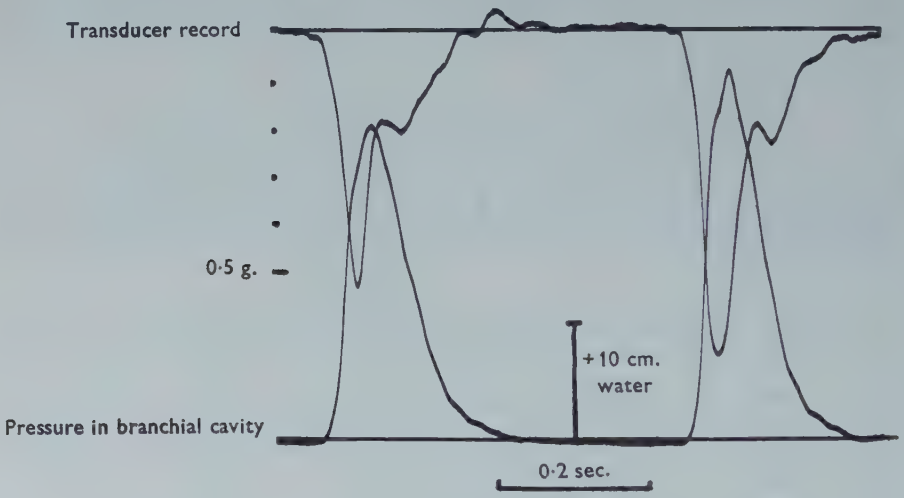

Fig. 11. *Anax*. A record of the pressure changes in the branchial chamber and the forces acting on the animal during swimming (upper trace).

Analyses made so far of pressure changes in the chamber are in agreement with the account given above. Recordings taken when a fine needle was inserted through the anus into the branchial chamber show a rapid rise in pressure at the onset of the longitudinal contraction and may reach pressures of about 30 cm. water (Fig. 11). The pressure falls to that of the surrounding water (taken as zero) as the abdomen begins to elongate. This fall may be even more rapid than the rise in pressure especially during contraction at the end of a series. The opening wide of the valve is presumably associated with this rapid fall and is succeeded by a very small negative pressure as water is drawn in. The positive pressure occurs simultaneously with the impulse on the insect and has a similar time course. Some preliminary attempts to record pressures in the body cavity suggest that the time course of these follows that of the longitudinal movements of the abdomen and not of the jet pressure or impulse records. Several other features of the pressure changes recorded during swimming as well as those occurring in respiration are of interest and will be considered in a later paper.

DISCUSSION

The importance of swimming in the life of these three insects varies. It is probable that *Dytiscus* swims the most and dragonfly nymphs the least. Both are carnivores but catch their prey in different ways. *Dytiscus* hunts actively in search of food (Tinbergen, 1951) but dragonfly nymphs lurk among weeds and await their prey. Swimming in the latter is mainly an escape mechanism for which rapid acceleration is of great importance. This is achieved by the expulsion of water at high speeds through a small aperture. The pressure and impulse records suggest that the jet velocity is about 250 cm./sec. and the aperture less than 0·01 mm.^{2} in cross-section. The same volume of water is drawn in through the anal opening, but by increasing the cross-section the velocity of the water relative to the animal is low and the consequent change of momentum retarding progression will be considerably reduced. The use of the same aperture for both the expulsion and entry of water is probably unique among animals which use this method of propulsion. In Cephalopods, for instance, water is drawn in through the lateral openings of the mantle cavity. The extreme rapidity and synchronization of activities at the beginning of dragonfly flight was described as 'die Gesamtreflex' by von Uexküll (1908) and the same term might well be applied to the swimming insect. Its co-ordination probably involves nerve fibres which run the whole length of at least the abdominal nerve cord. Some of these are relatively large (Hughes, 1953) and provide rapidly conducting pathways such as are frequently found in escape mechanisms.

Hydrophilus is a vegetarian and is the least modified both structurally and functionally. This is apparent in the movements of pairs of limbs which alternate in *Hydrophilus* but retract together in *Dytiscus* and *Anax*. It has been shown, however, that these alternating movements are not exactly the same as in walking because of differences in their nature and of the timing between the middle and hind pairs. It is of interest to note that in the larval stages of the beetles the opposite is true, for *Dytiscus* larvae 'trample' along with the two legs of a segment alternating whereas it is reported that those of *Hydrophilus* move together as the body performs dorso-ventral undulatory movements.

All three insects are adapted to swim along a straight path and their resistance to progression is reduced. Deviations from this path are corrected by a number of compensatory responses of the animal. The observations reported on the effects of limb amputation on *Dytiscus* indicate the extent of these in the normal animal, how they operate under modified circumstances and within limits produce the same end result. Thus although a precise analysis of any so-called plasticity change is not possible at a neurophysiological level, sufficient is now known of the extensive proprioceptive mechanisms of terrestrial insects to support the view that it is due to the altered stimulation of the proprioceptive mechanisms which occur when the mechanical conditions change (Hughes, 1957). The present work, while not ignoring such mechanisms, emphasizes the importance of extero-receptors in the orientation of aquatic animals where gravity does not have such a controlling influence. Of these the eyes are the most important, but there is some evidence that the antennae are important in detecting water currents as they are known to do in dragonfly nymphs (Tonner, 1935). The considerable importance of impulses reaching the central nervous system is shown in all these observations. The fineness of control which they exercise on motor responses probably involving extensive feed-back mechanisms, results in them often being taken for granted. The striking changes which occur following amputation, etc., show up their existence and give us some insight into their complexity. However, it must not be assumed that the original patterns of motor activity are completely discarded either in *Dytiscus* or in *Blatta*, for in both insects there remains a tendency for these to show up under 'stress'. Thus, if *Dytiscus* is dropped on water immediately after the amputation of a single hindleg, the remaining legs move rapidly and as a result the insect swims in small circles. But after a short time it slows up and makes a straighter path as described earlier. The same sequence is observed when the experiment is repeated on subsequent occasions even after a period of weeks.

SUMMARY

1. Movements of the legs during the swimming of adult *Dytiscus marginalis* and *Hydrophilus piceus* have been analysed from films. In both beetles the middle and hind pairs of legs play an active part and the front pair is held stationary beneath the thorax.

2. The two legs of a segment are retracted simultaneously in *Dytiscus*, the two limb pairs acting alternately. In *Hydrophilus* retraction of a hindleg is simultaneous with that of the contralateral middle leg. Movement is rectilinear in both insects, but the head of *Hydrophilus* rotates a little to the side opposite that on which the hindleg is retracting.

3. Following amputation of a single hindleg, *Dytiscus* swims along a relatively straight path as a result of modifications in the movements of the two middle legs and the remaining hindleg. Contrary to the description of Bethe & Woitas, the hindleg continues to alternate with the contralateral middle leg, the only change in rhythm being a delay in the retraction of the other middle leg. Some of the changes in action of these legs are similar to those used in turning of the normal insect and those which produce circling in unilaterally blinded insects.

4. Forced rotation of *Dytiscus* elicits compensatory movements which tend to produce circling in the opposite direction. The eyes, antennae and legs are the sites of receptors whose asymmetric stimulation produces this response. It is suggested that a similar asymmetry in the inflow from these sense organs is responsible for the modified movements following limb amputations.

5. In the swimming of nymphs of *Anax* the legs retract slightly before shortening of the abdomen takes place. Although all the segments take part, a large proportion of this shortening occurs in segments 6–8 which contain the branchial chamber. Water is ejected through a small aperture during this contraction but when water is drawn in the anal valves are open wide.

6. The pressure in the branchial chamber rises to about 30 cm. water within 0·03 sec. and the reaction from the jet enables the animal to attain speeds of 30–50 cm./sec. within the first centimetre of propulsion. The duration of this pressure and of the impulse on the animal correspond with the time during which the abdomen contracts longitudinally.

I wish to thank Dr Tybjaerg Hansen of the Rigshospitalet, Copenhagen, for allowing me the use of his electric manometers to measure pressure changes in the dragonfly nymph.

REFERENCES

AMANS, P. C. (1888). Comparaisons des organes de la locomotion aquatique. *Ann. Sci. Nat. Zool.* (sér. 7), **6**, 1–164.

BALDUS, K. (1927). Untersuchungen zur Analyse der Zwangsbewegungen der Insekten. *Z. vergl. Physiol.* **6**, 99–149.

BAUER, A. (1910). Die Muskulatur von *Dytiscus marginalis*. *Z. wiss. Zool.* **95**, 594–646.

BETHE, A. & WOITAS, E. (1930). Studien über die Plastizität des Nervensystems. II. Mitteilung. Coleopteren, Käfer. *Pflüg. Arch. ges. Physiol.* **224**, 821–35.

FAIVRE, E. (1857). Du cerveau des Dytisques considéré dans ses rapports avec la locomotion. *Ann. Sci. Nat. Zool.* (sér. 4), **8**, 245–74.

GRAY, J. (1953). Undulatory propulsion. *Quart. J. micr. Sci.* **94**, 551–78.

HANSEN, A. TYBJAERG (1949). Pressure measurement in the human organism. *Acta physiol scand.* **19**, suppl. 68.

HUGHES, G. M. (1952). The co-ordination of insect movements. I. The walking movement of insects. *J. Exp. Biol.* **29**, 267–84.

HUGHES, G. M. (1953). 'Giant' fibres in dragonfly nymphs. *Nature, Lond.*, **171**, 87.

HUGHES, G. M. (1957). The co-ordination of insect movements. II. The effect of limb amputation and the cutting of commissures in the cockroach (*Blatta orientalis*). *J. Exp. Biol.* **34**, 306–33.

KALMUS, H. (1936). Über die dynamische Erhaltung der Höhenlage durch die Sprungbewegungen der Larven und Puppen von *Corethra*. *Z. vergl. Physiol.* **23**, 651–62.

KALMUS, H. (1949). Optomotor responses in *Drosophila* and *Musca*. *Physiol. comp.* **1**, 127–47.

ROTH, W. (1909). Studien über konvergente Formbildung auf den Extremitäten schwimmender Insekten. II. Teil: Coleoptera. *Int. Rev. Hydrobiol.* **2**, 668–714.

SNODGRASS, R. E. (1954). The dragonfly larva. *Smithson. Misc. Coll.* **123**, 2, 1–38.

TINBERGEN, N. (1951). *The Study of Instinct.* Clarendon Press, Oxford.

TONNER, F. (1935). Schwimmreflexe und Zentrenfunction bei *Aeschna*—Larven. *Z. vergl. Physiol.* **22**, 517–23.

TONNER, F. (1936). Mechanik und Koordination der Atem- und Schwimmbewegung bei Libellenlarven. *Z. wiss. Zool.* **147**, 433–54.

TONNER, F. (1938). Halsreflexe und Bewegungssehen bei Arthropoden. *Z. vergl. Physiol.* **25**, 427–54.

VON UEXKÜLL, J. (1908). Studien über den Tonus. V. *Die Libellen*, **50**, 168–202.

WHEDON, A. D. (1918). The comparative morphology and possible adaptations of the abdomen in the Odonata. *Trans. Amer. Ent. Soc.* **44**, 373–437.

WIGGLESWORTH, V. B. (1953). *The Principles of Insect Physiology.* London: Methuen.

ZEISER, T. (1934). Die Aufhebung der Manegebewegung durch Photomenotaxis und negative Geotaxis beim Gelbrandschwimmkäfer *Dytiscus*. *Zool. Jb. Abt.* (3), **53**, 501–20.

THE EFFECT OF WEAK ELECTROLYTE SOLUTIONS ON THE HATCHING RATE OF THE EGGS OF *TRICHOSTRONGYLUS RETORTAEFORMIS* (ZEDER) AND ITS INTERPRETATION IN TERMS OF A PROPOSED HATCHING MECHANISM OF STRONGYLOID EGGS

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INTRODUCTION

During experiments to determine the optimum pH for the hatching of the eggs of *Trichostrongylus retortaeformis* (Wilson, 1957) it became evident that the concentration of the buffer solutions was an important factor determining the degree of hatch after a standard time. It seemed at first sight that this was a straightforward osmotic effect on the eggs, although the buffers used were not particularly strong solutions (range = 0·05–0·2 molar). This paper describes experiments which show that weak ionic solutions exert a specific effect on the hatching eggs which is not the consequence of colligative properties. Further investigations are described which attempt to use the ionic effect as a tool to elucidate the hatching mechanism.

The interpretation of the results is based on the structure of the strongyloid egg suggested by Monne & Hönig (1954). Their investigations indicate that the outer egg membrane is a quinone-tanned protein, the inner being a wax-like lipoid. They consider that the two membranes are not combined in any way, though they think that the lipoid of the inner layer may be supported by a tenuous protein skeleton.

There appears to have been no previous experimental attempt to discover the hatching mechanism of strongyloid eggs, though it seems generally to have been assumed that the first-stage larva simply forced its way out of the egg by its movements (e.g. Veglia, 1915, on *Haemonchus contortus*). Looss (1911), however, discounted the role of larval movement in *Ancylostoma duodenale* and considered hatching to be brought about by an increase in hydrostatic pressure in the egg fluid as a result of a change in the 'vitelline' (= 'lipoid') layer which rendered it semipermeable at the time of hatching. He based this conclusion on the fact that eggs could be more easily collapsed in strong salt solution when hatching was due to commence. Collapse of the contents of *Trichostrongylus retortaeformis* eggs in saturated sodium chloride shows, however, that the egg membranes are not semipermeable in the way Looss suggests—i.e. preferentially permeable to water—since only the larva is distorted under such conditions.

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MATERIALS AND METHODS

For this work it was important that as little development as possible should occur in the eggs between deposition in the hosts' faeces and subjection to the experimental conditions. Faeces from rabbits infected with *T. retortaeformis* were therefore collected every 2–3 hr. and stored in water at low temperature (2–4° C.) in containers with an efficient seal to prevent evaporation. Under these conditions development is effectively stopped and the eggs retain a viability of 70–80% for as long as 6 weeks (Wilson, 1957). In most experiments, however, the eggs used were obtained from faeces no more than a fortnight old.

Eggs were extracted from faeces by centrifugal-salt-flotation (Lane, 1928; McCoy, 1929), the flotation medium being a saturated solution of agricultural salt. Two centrifugations in the salt were usually necessary to remove sufficient of the faecal debris for the purpose of experiment, the eggs being thoroughly washed in distilled water before use. Eggs extracted in this way from fresh faeces showed a viability of 90–96%.

Hatching experiments were carried out at 30° C. in moist chambers to which air was accessible, or in sealed containers. The former were covered Petri dishes with the bottoms lined with moistened filter-paper, the eggs being placed in 0·2 ml. of the experimental solution in a watch-glass inside each Petri dish. In order to minimize exchange of water between watch-glass and filter-paper the latter was moistened with the same solution as contained the eggs, 2 ml. being used in every case. In this type of culture a certain amount of evaporation was inevitable, so that results obtained with it are qualitative only. According to availability and the requirements of the experiment in hand the sealed containers were various, the following being used at one time or another.

(*a*) Conical flasks of 250 ml. capacity with the eggs in 6 ml. or less of the experimental solution.

(*b*) 50 ml. conical flasks with the eggs in 1 ml. of solution (referred to later as 'flask cultures').

(*c*) Specimen tubes 3 × ½ in. containing the eggs in 0·1 ml. of solution ('specimen tube cultures').

(*d*) Two-drachm, screw-top, rubber-sealed bottles with the eggs in 0·2 ml. of solution ('bottle cultures').

The flasks were sealed with rubber bungs and the specimen tubes with waxed corks. The adequacy of the oxygen supply in the sealed vessels was assessed by comparison with the Petri dish moist chambers. Samples of 1300 eggs in distilled water showed no difference in hatch after 36 hr. at 30° C. in the different types of container. Nevertheless, precautions were often taken to minimize the risk of possible effects due to low oxygen tension in electrolyte solutions. Such precautions are indicated in the description of the experiments concerned.

Before introduction to the hatching containers the eggs were given one centrifugal wash in the experimental solution, being then distributed by graduated pipette in fresh solution to the cultures for incubation. For most experiments ten duplicate samples were set up for each treatment, approximately equal numbers of eggs in the samples of each treatment being ensured by a standardization of technique in any one experiment.

Estimates of the degree of hatch were made on cultures fixed in Gilson's fluid, the number hatched from a random sample of 50 or 100 being counted for each culture. These counts were made on micro-slides without a cover-slip, the stages being randomized by agitation with a bulb pipette washed in a very weak solution of 'Teepol'. Such a method was found to be necessary because the distribution of eggs and larvae is not random in a drop under a cover-slip (Wilson, 1957). The trace of 'Teepol' prevented the drifting of larvae that otherwise occurred.

EXPERIMENTS

(1) *Hatching of eggs in solutions of* NaCl *and sucrose*

Eggs were incubated in Petri dish moist chambers at 30° C. in concentrations of sucrose from 0–0·5 M and sodium chloride from 0·025–0·1 N. The stages were fixed in Gilson's fluid after 36 hr., estimates of the degree of hatch being made in the manner described. The mean hatch from the ten cultures for each concentration is plotted in Fig. 1 against the osmotic pressure of the solutions used.

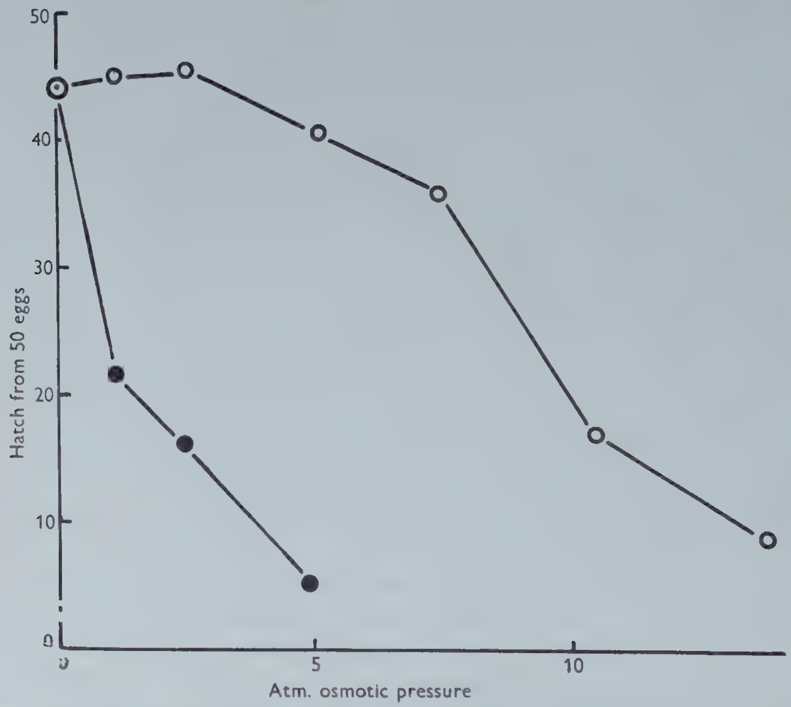

Fig. 1. Hatching of eggs of *T. retortaeformis* incubated in solutions of sucrose and NaCl for 36 hr. at 30° C. ○, mean hatch in ten sucrose cultures; ●, mean hatch in ten NaCl cultures.

The results show that both salt and sucrose in sufficient concentration reduce the number of eggs that hatch after 36 hr. at this temperature, but that the depression in salt solutions is manifest at concentrations where sucrose figures are little different from those in distilled water. Even if the influence of sucrose is not a straightforward osmotic effect, though it would seem likely that it is, the curves in Fig. 1 show that the effect of sodium chloride is not the consequence of colligative phenomena.

Eggs which had not hatched in either type of solution, nevertheless, contained fully developed and living first-stage larvae.

(2) *The extent of the depression of hatch by* NaCl

(*a*) *Ultimate hatch in* 0·05 N-NaCl *solution*

In order to determine whether sodium chloride permanently impaired the ability of some of the eggs to hatch, cultures were incubated in moist chambers in 0·05 N-NaCl and distilled water until maximum hatch had occurred, single cultures from the two series being fixed at intervals to determine the approximate rate of hatch. The results are plotted in Fig. 2*a*.

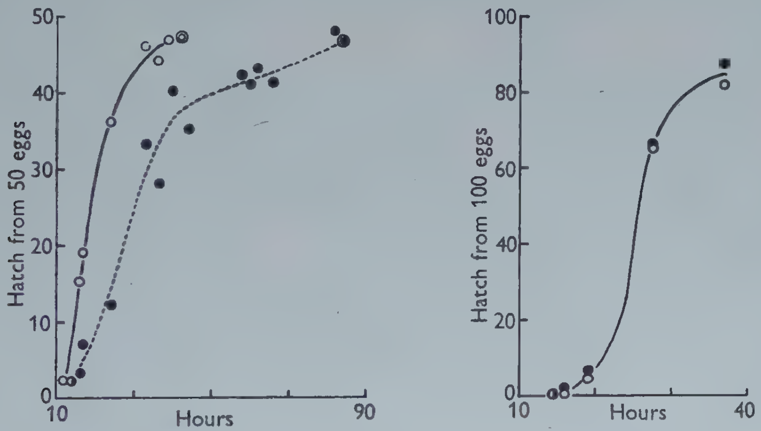

Fig. 2 (*a*)

Fig, 2 (*b*)

Fig. 2. (*a*) Approximate rate of hatch of *T. retortaeformis* eggs in distilled water (———) and 0·05 N-NaCl (- - - - -) at 30° C. ◎, mean of twenty cultures; ⊙, mean of sixteen cultures. (*b*) Rate of hatch of *T. retortaeformis* eggs in distilled water after 12 hr. in 0·05 N-NaCl (●) or distilled water (○). Each point is the mean of ten cultures.

The final mean hatch from fifty eggs in the two media was 47·3 (distilled water —twenty cultures after 42 hr.) and 46·7 (NaCl—sixteen cultures after 83 hr.), thus demonstrating that sodium chloride reduces the rate of hatch without affecting the final 'hatchability' of the eggs. The results also suggested that processes of development in the egg before hatching are not slowed down since hatching apparently started at the same time in both salt solution and distilled water.

(*b*) *Influence of* 0·05 N-NaCl *on development before hatching*

The preceding results showed that hatching normally commenced after about 12 hr. incubation at 30° C. Eggs were therefore incubated for this time in distilled water and 0·05 N-NaCl, being then thoroughly washed and distributed in Petri dish moist chambers in distilled water to discover the effect of prior incubation in NaCl. Ten cultures from each series were fixed simultaneously at intervals and the mean hatch from 100 eggs estimated. The results (Fig. 2*b*) show that the rate of hatch of eggs incubated initially in NaCl was not slower than the controls. Thus the depressing influence of sodium chloride on hatching is one concerned with the hatching mechanism itself, processes of development up to this stage not being slowed down.

(3) *The hatching of eggs in various electrolytes*

The results of experiments with buffer solutions referred to earlier suggested that an effect similar to that of sodium chloride on hatching rate would also be exerted by other ionic solutions. To confirm this, eggs were incubated in 0·05 N solutions of the following: CH_3COONa, Na_2SO_4, $NaCl$, K_2SO_4, KNO_3, KI, Li_2SO_4, $CaCl_2$ and $MgCl_2$.

Ten cultures for each of these solutions were incubated simultaneously in specimen tubes (see earlier) after an initial 10 hr. in distilled water. The preliminary incubation was carried out to shorten the time during which free oxygen was inaccessible to the eggs. After a total of 36 hr. (10 in distilled water and 26 in the ionic solutions) the contents of the tubes were fixed and counted. Controls were run simultaneously in 0·1 M. sucrose in order to obtain a measure of any purely osmotic effect.

The mean hatch from fifty eggs in each electrolyte is plotted in Fig. 3 against the equivalent conductance of the pure solutions at 25° C. (Harned & Owen, 1950). The highest mean hatch (25·0/50 in sodium acetate) was very much below that for the controls in sucrose (44·0/50), so that, clearly, all the ionic solutions reduced the rate of hatch to a significant degree. In addition it can be seen that a correlation exists between the depression in hatch and the conductance of the solutions, ($r = -0{\cdot}895$ for 7 D.F., $P < 0{\cdot}01$). Thus the depression in hatch is greater in solutions of ions with higher mobilities.

Since in general the permeability of a membrane to ions is proportional to the ionic mobilities the results suggest that the depression in hatch is related to the concentration of electrolyte that is able to penetrate the egg membrane in a given time. The question arises as to whether the cation or the anion is the more important constituent on the assumption that such penetration takes place. The results plotted in Fig. 3 may be analysed in terms of the mobility of the ions separately. If this is done a significant relationship ($P = 0{\cdot}02$) is found to exist between hatch and the mobility of the cation (see Table 1).

Although the value of r for the anion-hatch relationship is not significant ($P = 0{\cdot}08$) it does indicate that 39% of the variation can be attributed to the effect of the anion. Since, on the whole, the cation was slower than the anion in each of the electrolytes used (CH_3COONa and KNO_3 were the exceptions), the correlations in Table 1 suggest that the slower ion in any one salt is the factor determining its influence. Calculations of r for the relationships slow-ion/hatch and fast-ion/hatch show clearly that this is the case, the value of the former being significant ($P < 0.01$), that for the latter being very small and indicating an influence on the variation of

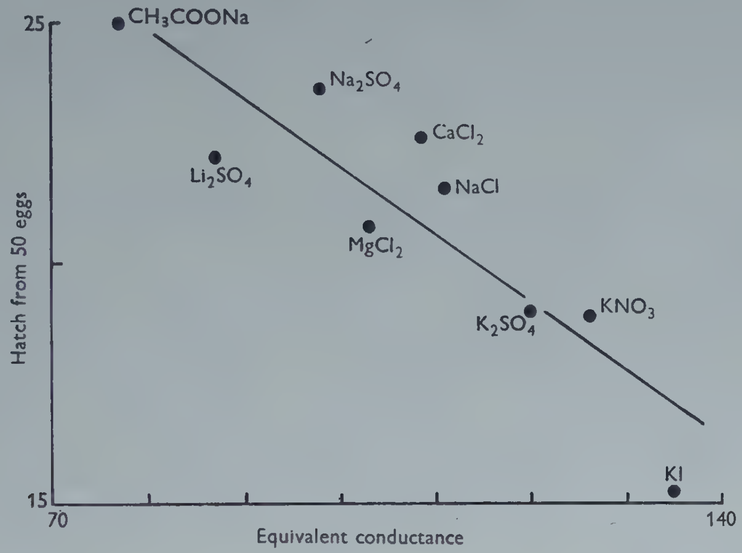

Fig. 3. Degree of hatch of *T. retortaeformis* eggs incubated in 0·05 N solutions of different electrolytes at 30° C. over the normal hatching period. Each circle is the mean of ten cultures. Control hatch in 0·1 M sucrose = 44·0.

Table 1. *Relationship between hatch (z) and the mobility of the cation (x) and anion (y) in each salt*

Relationship	r	Significance	Approximate % variation accounted for
x and z	−0·768	0·02 P	59
y and z	−0·623	0·08 P	39
x and y	+0·235	—	6

Table 2. *Relationship between hatch (z) and the mobilities of the faster ion (x) and the slower ion (y) in each salt*

Relationship	r	Significance	Approximate % variation accounted for
x and z	−0·079	—	0·6
y and z	−0·846	0·01 P	72
x and y	+0·510	—	26

less than 1% (see Table 2). The remaining variation can be attributed to a tendency for the fast ions to be associated together, though r for this relationship ($+0\cdot510$) is not significant.

Thus, while the depression in hatching rate is greater in solutions of ions with higher mobilities (Fig. 3), the effect of any one electrolyte is controlled by the speed of its slower ion irrespective of whether it is the cation or the anion. It follows from this that the effect is not an 'exchange' phenomenon in the first instance, but is the consequence of the penetration of the egg membrane(s) by both components of a salt. A further corollary is that other specific effects of different ions on the hatching mechanism are relatively unimportant, otherwise the correlation with mobility would be destroyed.

(4) *The effect of* 0·05 N-NaCl *on the water permeability of hatching eggs*

(*a*) *Water permeability of normally hatching eggs*

It has been stated by Looss (1911) that the contents of the eggs of *Ancylostoma duodenale* are more easily collapsed in strong salt solution at the time they are due to hatch. If it were found that the eggs of *Trichostrongylus retortaeformis* behave similarly this would suggest that ions exert their effects when shrinkage—i.e. water permeability—is at a maximum. The normal behaviour of eggs in this respect was therefore examined by transfer of samples of eggs incubated in distilled water to strongly hypertonic salt and sucrose solutions. (The salt solution and sucrose solution were isotonic and were compared so that any differences in their effects might be revealed.)

Eggs were incubated in a sealed 250 ml. conical flask in distilled water, samples being removed by pipette at intervals to determine the rate of shrinkage. This was done in the following way. The eggs from the pipetted sample were spun down to the bottom of a conical centrifuge tube, the supernatant was removed, and the tube was part-filled with hypertonic solution, either 2 molal NaCl, or 3·05 molal sucrose, each having an osmotic pressure of 96·2 atm. (the strength of these solutions was calculated from data supplied by Robinson & Stokes, 1949). The eggs were thoroughly mixed with the hypertonic solutions, samples were removed at intervals over a period of an hour or so and the number collapsed from a random group of fifty was recorded for each sample.

The results from the two solutions agreed in that no shrinkage occurred until just before hatching was due to commence, even if the eggs were left in the solutions for as long as 12 hr. Just before hatching commenced a proportion of the eggs became permeable to water and this proportion increased as hatching progressed.

The detailed behaviour of the eggs in the two solutions was different, however, after 13 hr. incubation. This difference was best illustrated by the 14 hr. sample, the curves for which are plotted in Fig. 4. In general, the number of eggs collapsed in sucrose solutions showed a straight line increase with time. In the salt solutions, on the other hand, there were three clearly defined components making up the total curve, namely: (*a*) an initial rapid increase in shrinkage followed by (*b*) a constant phase over which no change was apparent (within the limits of the sampling error), and a final phase (*c*) in which further increase occurred.

Despite these differences the results show that the hatching eggs of *T. retortaeformis* conform roughly to the pattern referred to briefly by Looss for *Ancylostoma duodenale.* In the present experiments, however, a much more marked change was noted than that indicated by Looss, since it was impossible to damage healthy eggs during the prehatching developmental period—indicating that they are completely impermeable to water at this stage.

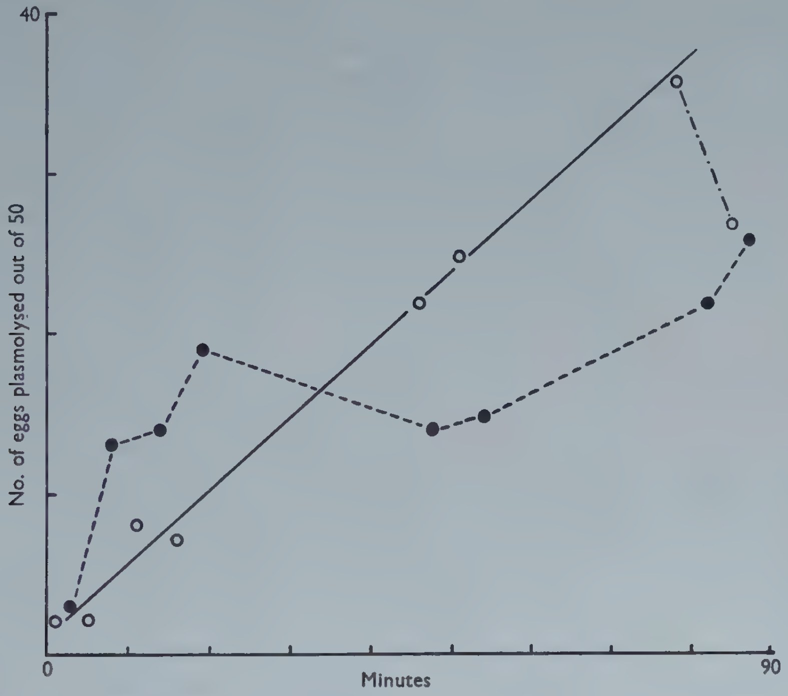

Fig. 4. Collapse of eggs of *T. retortaeformis* in isotonic sucrose (○) and NaCl (●) solutions after 14 hr. incubation at 30° C. in distilled water.

In comparing the effects of the two solutions certain errors must be taken into account which are inherent in the use of sucrose. First, it is a simple matter to identify a water-permeable egg in NaCl since the contained larva is almost immediately affected, the egg membranes retaining their shape in most cases. In sucrose, however, the initial stages of collapse are difficult to detect because the egg membranes contract slowly against the contours of the still apparently normal larva, so that the orientation of the egg plays a part in whether the effect can be seen. Unless the egg is positioned so that the head or the tail of the larva can be seen at the side the distortion of the shell cannot be detected. In addition, the final effects of hypertonic sucrose are so destructive that it is difficult to identify the remnants as eggs at all. For these reasons the number of collapsed eggs may be underestimated and it may even appear that there has been a decrease in water permeability (e.g. the last point on the sucrose curve in Fig. 4). The main differences between the two curves cannot, however, be attributed to these errors since, even with the underestimation mentioned, the figures for sucrose eventually exceed those for salt. The probable significance of these differences is referred to in the discussion.

(*b*) *Water permeability of eggs incubated in* 0·05 N-NaCl

With the normal picture in mind experiments were carried out to discover departures from the normal shown by eggs developing in a weak NaCl solution. Eggs were incubated in bottle cultures (see earlier) in 0·05 N-NaCl and in distilled water, bottles from the two series being removed at intervals and the degree of shrinkage estimated after 10 min. in a hypertonic solution on a slide.

After a sample had been removed from each bottle for the water permeability estimation the contents remaining were fixed in Gilson's fluid for later estimation of the degree of hatch at that time to be made. Two separate experiments were conducted, one using a strong sucrose solution (approximately 1·5 M), the other a near-saturated solution of NaCl. The time of immersion in the hypertonic media was 10 min.; the results therefore relate to the horizontal part of the NaCl curve of Fig. 4.

The results of these experiments are plotted in Fig. 5 *a–d*, the estimates of hatch being the means of five counts from each bottle culture. Despite the detailed differences between the curves for salt and sucrose the results agree in showing a lower level of shrinkage in eggs incubated in 0·05 N-NaCl. This is the opposite of what might be expected on the basis of the idea earlier expressed—i.e. that the ions exert their effect at the time when the egg becomes permeable to water. If this idea had been correct shrinkage would have been greater in eggs from the weak NaCl cultures.

Two further possibilities remain depending partly upon whether water permeability is a necessary prerequisite for hatching, and partly on whether ions in solution specifically affect the attainment of water permeability. These possibilities are (*a*) that the hatching mechanism as a whole is slowed down, or (*b*) that the low shrinkage level indicates a specific effect of ions on water permeability. If water permeability was essential for hatching—and not, as it may well have been, a mere consequence—and if it was inhibited by ionic solutions, then such inhibition could perhaps have accounted for the delayed hatching rate.

The results plotted in Fig. 5 *a–d* can be used to determine whether 0·05 N-NaCl has any specific effect on water permeability by calculating the 'residual' and 'total' water permeabilities for each point on the distilled water and 0·05 N-NaCl curves. In order to do this the hatching and shrinkage figures must be related in some way. According to the original method these properties were estimated on separate samples, there being no direct connexion between the figure for hatching (number hatched out of 50) and that for shrinkage (number collapsed out of 50 *unhatched* eggs). It can safely be assumed that those eggs that had hatched became permeable to water before doing so. There remains a number of unhatched eggs (i.e. $50-h$, where h is the number hatched from 50) of which $(50-h)p/50$ are permeable to water (where p is the number collapsed out of 50 in Fig. 5*a*–*d*). The value of $(50-h)p/50$ is termed the 'residual permeability' and represented by p'. The 'total permeability' for each point then becomes $(p'+h)$, since in this connexion the hatched eggs are regarded as permeable to water.

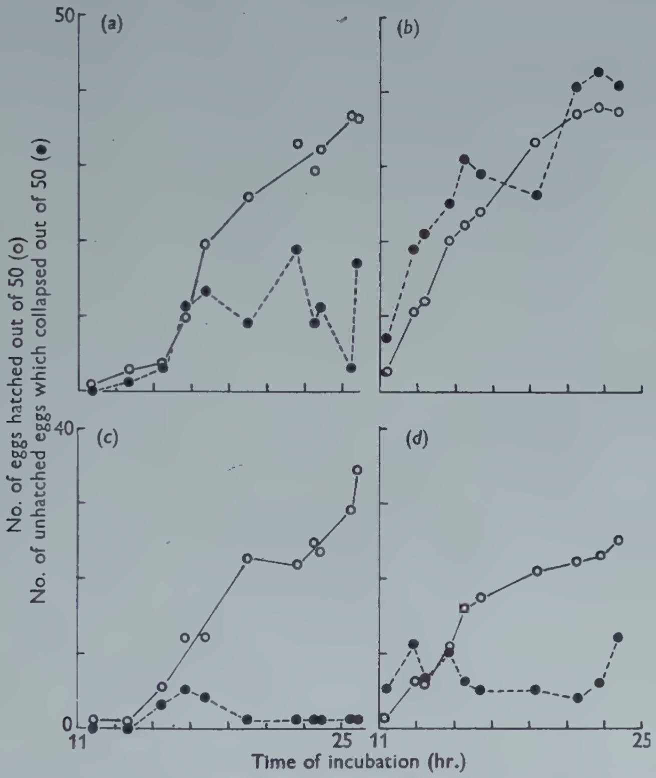

Fig. 5. Water permeability of *T. retortaeformis* eggs hatching in distilled water $(a+b)$ or 0·05 N-NaCl $(c+d)$ at 30° C. Permeability to water measured by collapse in 1·5 M sucrose $(a+c)$ and saturated NaCl $(b+d)$.

Now, if the effect of ions on hatching rate is a general slowing down of all processes, of which change in water permeability is a consequential or necessary part, then the ratio $p'/(p'+h)$ should be the same for eggs hatching in either 0·05 N-NaCl or distilled water. If, on the other hand, ions exert a specific depressing effect on water permeability, then one would expect the value of $p'/(p'+h)$ to be significantly lower in eggs from 0·05 N-NaCl.

When these calculations are made the figures for collapse both in salt and in sucrose provide the same answer, though only those for salt are represented in Fig. 6. Except for one pair of points they show clearly that $p'/(p'+h)$ is significantly lower in eggs from the 0·05 N-NaCl cultures. Thus it would appear that the change in permeability to water is specifically inhibited by ions in solution. The fact that over the period where maximum hatching rate is attained in distilled water the number of eggs permeable to water in 0·05 N-NaCl stays at a constant low level (Fig. 5*c*, *d*) supports the view that this is in fact the means whereby hatching rate is slowed down in ionic solutions. It would follow from this that the hatching rate of eggs in distilled water is controlled by a 'second process' which normally lags behind the change in water permeability and which is not so markedly affected by ions in solution. The possible nature of this process is dealt with in the discussion.

Since it is highly probable that water permeability is controlled by the lipoid layer of the egg it is possible that the effect of ions is concerned with the stabilization of this membrane beyond a point when it would normally be broken down.

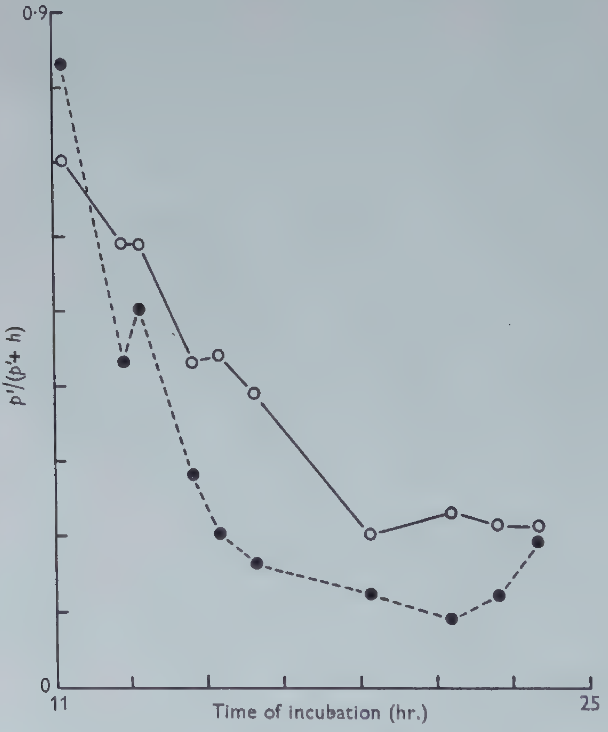

Fig. 6. 'Permeability ratio' of *T. retortaeformis* eggs incubated in distilled water (○) and 0·05 N-NaCl (●) at 30° C. Calculated from the data in Figs. 5*b*+*d* (see text).

(5) *Compounds which antagonize the effect of ions on hatch*

(*a*) *'Teepol'*

This possibility suggested the investigation of the effect of electrolytes on hatching in the presence of small concentrations of the strong emulsifying agent, 'Teepol'.

Eggs were incubated in Petri dish cultures of 0·05 N-NaCl with concentrations of $1/10^6$, $1/10^5$ and $1/10^4$ by volume of 'Teepol', the mean hatch from the ten cultures for each treatment being estimated on material fixed after 36 hr. incubation. Before fixation, however, the eggs and hatched larvae were examined to see if 'Teepol' was toxic. Although no numerical estimate was made, there was an obvious poisoning effect of the 'Teepol' at its highest concentration, since more than the usual number of eggs had died before the larvae were fully developed. Nevertheless, all the cultures containing 'Teepol' showed a higher hatch than the culture in 0·05 N-NaCl alone, the means for the two higher 'Teepol' concentrations being significantly greater at $P=0·05$ (Table 3).

Table 3. *Effect of 'Teepol' on hatching in* NaCl *solution*

Mean hatch from fifty eggs in				
0·1 M sucrose	0·05 N-NaCl	0·05 N-NaCl+'Teepol' at the following volume concentrations		
		10^{-6}	10^{-5}	10^{-4}
45·5	15·3	20·8	26·7	23·0

(*b*) *Non-emulsifying compounds*

A simple theory may be deduced from the above experiment which relates the effect of 'Teepol' to its ability to emulsify the lipoid membrane in opposition to an hypothetical stabilization by ions in solution. However, the position is complicated by the fact that a number of other substances having little or no emulsifying power show a similar, or greater, antagonism to the effect of ions on hatching. Thus, the addition of the first three alkyl acetates at a concentration of 0·01 M to the 0·05 N-NaCl solution produces a hatching curve very similar to that obtained by simply increasing the 'Teepol' concentration (Table 4).

Table 4. *Effect of alkyl acetates on hatching in* NaCl *solution*

Mean hatch from fifty eggs in				
0·1 M sucrose	0·05 N-NaCl	0·05 N-NaCl+0·01 M-CH_3COOR, $R=$		
		CH_3	C_2H_5	C_3H_7
39·8	7·3	10·1	13·8	12·4

Table 5. *Hatching of eggs in mixtures of glycine*, NaCl *and* NaOH

Estimate	Hatch from fifty eggs (mean of ten cultures) in				
	Distilled water	Glycine buffer at pH			
		8·40	9·20	9·58	10·30
1	46·8	39·2	—	33·1	37·6
2	—	32·0	33·5	—	38·5

Again, the high hatch obtained in glycine buffer solutions (Table 5) is remarkable when one considers that they contain NaCl up to a concentration of 0·1 M. Efforts to identify the salt-antagonizing agent in these solutions showed that the effect was one concerned with the properties of the mixture, and was not due to the individual influence of glycine, NaOH or pH.

Evidence also suggests that sucrose can act as an antagonist to the effect of ions on the hatching eggs.

(6) *Examination of the role of water permeability in the hatching egg*

(*a*) *Size of the egg and unhatched larva before and after the change in water permeability occurs*

Since the egg in its natural environment is almost certainly surrounded by a medium hypotonic to its contents it seems highly probable that osmotic uptake of water will take place as soon as it becomes permeable. The increase in hydrostatic pressure that results may be discernible in an increase in size, either of the egg itself, or of the contained larva, or of both.

It has been shown that delayed hatching in ionic solutions is probably the consequence of the inhibition of the change in water permeability, so that osmotic uptake of water may possibly be part of the hatching mechanism even though it cannot occur in the way Looss suggested (i.e. via the *egg* membranes, see introduction).

To examine this possibility the length and breadth of the egg and the width of the unhatched larva were measured in 0·1 M sucrose before and after change in permeability had occurred. Controls were incubated and measured in 0·05 N-NaC in which water permeability stayed at a minimum throughout. The mean sizes of thirty eggs or unhatched larvae from the different media are to be found in Tables 6*a* and *b*.

Analysis of variance shows that no significant change in size was apparent either in NaCl or in sucrose. This does not necessarily mean that no increase in hydrostatic pressure has occurred since, in the first place, the protein egg shell may be sufficiently rigid to show no distortion until breaking point is reached, and in the second place the larva is already closely confined in the egg, so that no significant increase in size need be manifest even though uptake of water raises the hydrostatic pressure. On a matter of technique, the error-variance for the measurements of the width of unhatched larvae was considerable and may easily have masked any effect that may possibly have occurred. In contrast, the egg dimensions could be more accurately determined, so that it is unlikely that any change in size did in fact occur in this case.

(*b*) *Movement of the unhatched larva just before hatch*

If the idea that hydrostatic pressure within the larva increased after the egg became permeable to water is true, then it seems likely that movements of the larva will become more restricted when hatching is imminent.

Table 6*a*. *Influence of water permeability on the size of the eggs*

Incubation (hr.)	No. of eggs that collapsed out of fifty in sat. NaCl		Mean size of thirty eggs in			
			0·05 N-NaCl		0·1 M sucrose	
	NaCl	Sucrose	Length	Breadth	Length	Breadth
11	Nil	Nil	63·3	35·8	64·0	35·4
24½	1	46	63·8	35·9	61·9	34·6

Units are mm. apparent size of the camera lucida drawing (absolute size 85–91 × 46–56 μ).

Table 6*b*. *Influence of water permeability on the width of unhatched larvae*

Incubation (hr.)	No. of eggs that collapsed out of fifty in sat. NaCl		Mean width of thirty larvae in	
	NaCl	Sucrose	0·05 N-NaCl	0·1 M sucrose
11	Nil	2	13·1	13·2
24½	3	48	13·3	13·5

Units are mm. apparent size of the camera lucida drawing.

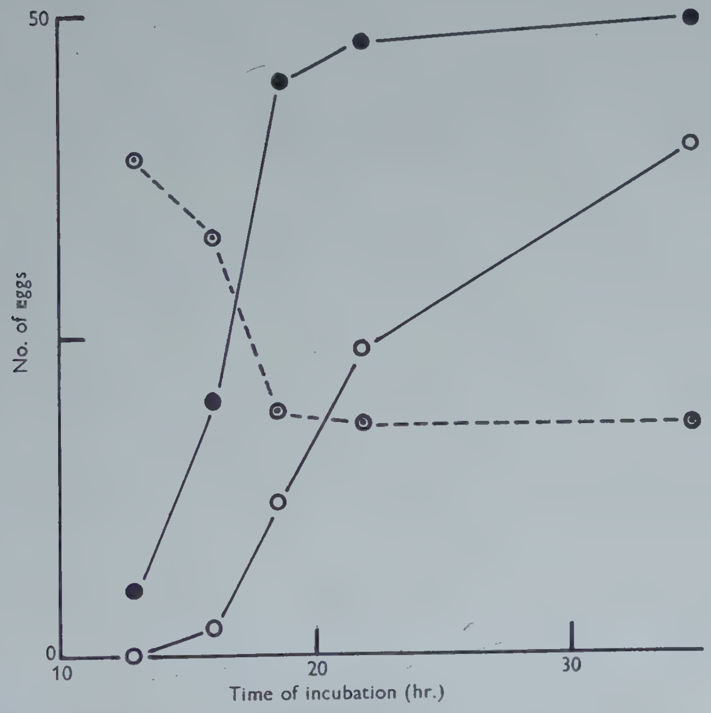

Fig. 7. Water permeability of the eggs (●), hatch (○) and mobility of unhatched larvae (⊙) of *T. retortaeformis* incubated in distilled water at 30° C. Estimates of water permeability made by plasmolysis in sat. NaCl.

Eggs were incubated in distilled water and the following properties were estimated on samples removed by pipette during the hatching period:

(*a*) degree of hatch from fifty eggs,

(*b*) the number of unhatched larvae out of fifty that would collapse in saturated salt solution, and

(*c*) the number of eggs out of fifty that contained mobile larvae when first observed.

Conditions for (*c*) were standardized as far as possible, though temperature control on the microscope stage could not be attained. Oxygen concentration was adequate throughout since the hatched larvae showed high activity.

The results (Fig. 7) show that movement decreased as hatching progressed, but, more important, mobility was at a maximum when permeability was at a minimum, and vice versa, the mobility curve being a mirror-image of the permeability curve. The results suggest that the larva is unable to move so freely just before hatching, and that this is due to the osmotic uptake of water.

INTERPRETATION AND DISCUSSION

Despite differences of shrinkage in saturated salt solution and strong sucrose solution, such experiments indicate that water permeability is essential for hatching in *Trichostrongylus retortaeformis* eggs, the specific inhibition of the change in water permeability by weak electrolyte solutions being sufficient to slow down the rate of hatch. Thus the breakdown of the lipoid layer as a water-resisting barrier appears to be an obligatory part of the hatching mechanism, though, under normal conditions, it proceeds at a rate faster than the final processes and therefore does not control the rate of hatch.

The conclusion that the rate of lipoid breakdown can be made to control hatching rate in ionic solution is supported by the partial antagonizing influence of an emulsifying agent such as 'Teepol', though the effect of various other materials in this respect cannot be explained.

In deducing the means whereby permeability to water is achieved it would seem probable that the movements of the larva are important. From the 'tadpole' stage onwards these movements become increasingly obvious, until, just before the egg becomes permeable to water, the larva can be seen gliding round and round inside its confining membrane. Such agitation of the egg fluid by the larva would assist emulsification of the inner egg layer; though the presence of some emulsifying agents in the fluid must be presupposed.

It seems likely that once the lipoid is broken down as a water-resisting barrier the difference in osmotic pressure between the external medium and the larval fluids hypertonic to it causes the larva to take up water from the surroundings and thereby to exert further pressure on the protein shell. The view that this occurs is supported by the fact that larval movements become more restricted after the egg becomes permeable to water, though no measurable swelling occurs at the same time.

It has already been explained that the figures for hatching and permeability in distilled water indicate that at least one other process is involved in hatching besides that dependent on water permeability. This process apparently controls hatch under normal conditions and is not slowed down to the same extent in electrolyte solutions. Qualitative observations of hatching eggs suggested that this second process is some sort of chemical weakening of the outer protein coat, though the more obvious experiments to test this hypothesis have proved negative. It has been found that hatching rate of eggs in samples with average numbers per sample ranging between 250 and 6750 is the same— i.e. there is no evidence for the autocatalysis of hatching eggs. In addition the supernatant from eggs that have reached maximum hatching rate does not appear to accelerate the hatching of other eggs immersed in it. If, therefore, a 'hatching factor' is secreted it must be a compound which is easily broken down once released from the egg. There is slight evidence which is being further studied that such an unstable substance is produced, but it is too tenuous to be discussed in detail here.

The evidence reported here suggests, however, that at least two distinct processes are involved in the hatching of the eggs of *T. retortaeformis*, one depending upon the breakdown of the inner lipoid membrane of the egg and increase in hydrostatic pressure inside the larva, and another subsequent process which normally controls the rate of hatch in the absence of ions in solutions. It is suggested that the first process is brought about by the agitation of the egg fluid by the larva, with the assistance of an emulsifying agent, and that its function is to enable the larva to exert pressure on the rigid protein shell. The second process is thought to be some sort of chemical weakening of the protein shell, which, in combination with the pressure resulting from the first process, enables the larva to escape.

With the possible exception of the Metastrongylidae it would seem likely that members of the Strongyloidea in general would share the same hatching mechanism as is found in the Trichostrongylidae, in view of the similarities in this part of the life-cycle of most species of the order.

The problem of exactly how the breakdown of the lipoid membrane is affected by ions in solution remains to be investigated. Speculation about the possible penetration of the shell layers poses the question as to whether control of the rate of penetration is exerted by the wax-like layer or the protein one.

At first sight it would seem likely that the effect of the ions would be to alter the egg fluid surrounding the larva—i.e. after the penetration of both shell layers. This, however, requires the wax membrane to be permeable to ions just before hatching when it would still be impermeable to water (since the main effect of salts is to delay the change in water permeability). Such a system is not impossible but appears to be rarely, if ever, encountered in biological studies. The sort of membrane required would have to be non-porous and to contain carrier molecules with an affinity for ions of the same order as the affinity of water for ions. The main objection to this theory is the fact that during development of the embryo the membrane is impermeable to ions as well as to water, otherwise plasmolysis would occur in saturated salt simply as a result of the penetration of ions. As has been indicated, developing eggs can be left in hypertonic salt solutions for as long as 12 hr. without apparent harm. Thus, one is faced with the problem of how the carriers become modified at the time when the breakdown of the lipoid commences, since before this they appear to be incapable of ion transfer.

The difficulties of interpretation are less if one assumes that the effect is exerted between the protein and lipoid layers, either directly on the outer surface of the latter, or indirectly by an effect on an interlayer material instrumental in lipoid breakdown. The effect of ion mobility would then be an indication of the relative ion penetration through the protein shell.

The exact influence on the inner egg layer is still problematical, of course, though any explanation must be based on the apparently uniform effect of different ions and the fact that the charge on the ion plays an important part—for instance $\frac{1}{2}SO_4^{2-}$ appears to have an effect comparable with that of Cl^- (see Fig. 3).

The general idea that breakdown of the wax membrane is delayed by ions is supported by the differences in behaviour of eggs in hypertonic salt and sucrose solutions (see Fig. 4), the 'constant phase' of the salt curve being produced by this effect. These results suggest also that sucrose accelerates breakdown of the inner layer, thus supporting direct evidence of sucrose antagonism to the ion effect.

The possible adaptive significance of the effect of ions on hatch can be considered in the light of the well-known fact that the water content of the faeces of many host animals is insufficient to promote full development of strongyloid stages. Under field conditions the faeces may be subject to desiccation for some time after they are deposited, further lowering the chances of survival of the delicate first-stage larvae.

If the larva were retained in the egg under these conditions, and the lipoid coat remained intact as a water-resisting barrier, then its chances of survival would proportionately increase.

A circumstance that would follow from loss of water from the faeces would be an effective increase in the concentration of substances in solution in the faecal fluid, including any electrolytes that may be present. Thus the larva would be retained in the egg for a period inversely proportional to the water content of the faeces if the electrolyte concentration of the medium reached a significant level. Moistening of the faeces by rain or dew would dilute the faecal solutions and hasten the escape of the larva.

This must be set against the fact that various materials are able to antagonize the action of electrolytes, and some of these may be present in the faecal fluid. Only in the case of glycine buffer solutions was there any approach to normal hatching rate, however. Thus it is possible that this salt-antagonizing effect is not so important a factor under field conditions as is the concentration of electrolytes.

SUMMARY

1. The influence of solutions of NaCl on the hatching of eggs of *Trichostrongylus retortaeformis* is studied. It is shown that the effects are not the consequence of colligative properties, but are related to ionic phenomena. 0·05 N-NaCl slows down

the rate of hatch without impairing the ultimate 'hatchability' of the eggs. Processes of development up to hatching are not slowed down.

2. The effect demonstrated in the case of NaCl is shown to be shared by eight other electrolytes, the depression in the rate of hatch being proportional to the mobility of the ions in solution. On the assumption that the effect of the ions is due to a penetration of the egg membrane(s) the rate of entry is shown to be controlled by the speed of the slower ion in any one salt.

3. The influence of NaCl on the permeability of hatching eggs to water is studied. It is shown that the rate of increase in permeability is slowed down sufficiently in NaCl to control the rate of hatch. The inference that water permeability is a necessary prerequisite for hatching is made, a further hypothetical process being invoked to account for the rate of hatch in the absence of NaCl, since it is not then controlled by changes in water permeability.

4. The probability that the net effect of ionic solutions on the eggs is one concerned with the rate of breakdown of the inner wax-like layer of the egg is strengthened by experiments demonstrating that the depressing influence of NaCl is antagonized by 'Teepol', though the comparable influence of other, non-emulsifying, compounds cannot be explained.

5. The role of water permeability in the hatching mechanism is investigated.

6. A hatching mechanism of strongyloid eggs is proposed which involves two processes, the first dependent upon the osmotic relationships of the unhatched larva to its environment, the second being some sort of chemical weakening of the outer shell.

7. It is suggested that the effect of ions on hatching rate assists the 'embryonated egg' to survive under natural conditions when the hatched first-stage larva might otherwise be destroyed by desiccation.

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REFERENCES

Harned, H. S. & Owen, B. B. (1950). *Physical Chemistry of Electrolytic Solutions.* American Chemical Society Monograph Series, no. 95. New York: Reinhold Publishing Corporation.

Lane, C. (1928). The mass diagnosis of hookworm infection. *Amer. J. Hyg.* 8 (suppl.) 1–148.

Looss, A. (1911). The anatomy and life-history of *Anchylostoma duodenale* Dub. Part II. *Rec. Egypt. Govt. School Med.* **4** (Monograph, 446 pp.).

McCoy, O. R. (1929). The suitability of various bacteria as food for hookworm larvae. *Amer. J. Hyg.* **10**, 140–56.

Monne, L. & Hönig, G. (1954). On the properties of the egg envelopes of various parasitic nematodes. *Arkiv Zool. (Stock.)*, **7** (3), 261–72.

Robinson, R. A. & Stokes, R. H., (1949). Osmotic and activity coefficients of electrolytes in aqueous solution at 25° C. *Trans. Faraday Soc.* **45**, 612–24.

Veglia, F. (1915). The anatomy and life-history of the *Haemonchus contortus* (Rud). *3rd and 4th Reports of the Director of Vet. Res.* Pretoria, S. Africa, pp. 349–500.

Wilson, P. A. G. (1957). Studies on the physiology and behaviour of the immature stages of *Trichostrongylus retortaeformis* (Zeder). Ph.D. thesis, University of Leeds.

TIDAL AND DIURNAL RHYTHMS OF LOCOMOTORY ACTIVITY IN *CARCINUS MAENAS* (L.)

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INTRODUCTION

Since the early observations of tidal rhythms in *Convoluta* (Gamble & Keeble, 1903, 1904) and in *Actinia* (Bohn, 1906) much experimental work on rhythms of behaviour in intertidal animals has been concerned with such phenomena as oxygen uptake and colour changes. Gompel (1937) demonstrated tidal rhythms of oxygen consumption in *Patella*, *Mytilus* and *Haliotis* and similar rhythms have been shown more convincingly in several other organisms by F. A. Brown and his co-workers. Superimposed tidal and diurnal rhythms of oxygen consumption are described in *Littorina littorea* and *Urosalpinx cinereus* (Sandeen, Stephens & Brown, 1954), and in *Uca pugnax* and *U. pugilator* (Brown, Bennett & Webb, 1954), and similar rhythms of colour change occur in *U. pugnax* (Brown, Fingerman, Sandeen & Webb, 1953) and *Callinectes sapidus* (Fingerman, 1955). Along similar lines, Rao (1954) has described persistent tidal rhythmicity in the rate of water propulsion by *Mytilus*. Many physiological rhythms of this kind have been reported but, as has been noted by Bennett, Shriner & Brown (1957) several are apparent only after statistical analysis of extensive data.

Despite the fact that it is often more simple to obtain a continuous record of the movement of an animal than of its oxygen uptake there are few published examples of rhythms of locomotory activity in marine animals. Recent demonstrations of tidal rhythms of this nature in *Nassa obsoleta* (Stephens, Sandeen & Webb, 1953) and *Uca pugnax* (Bennet *et al.* 1957) are notable. The present paper describes spontaneous tidal and diurnal rhythms of locomotory activity in *Carcinus maenas* (L.)

METHODS

Two types of apparatus were used, one in which a crab was kept moist in air and another in which it was continuously immersed in sea water. The first type consisted of a box measuring 10 cm. wide × 15 cm. long × 4 cm. high, constructed of thin transparent Perspex and suspended in pivots at the centre of each long side. The inside of the box was moistened with sea water and a single specimen of *Carcinus* enclosed. The box was allowed to tilt slightly when the crab moved across the axis of suspension and a wire stylus attached beneath the box recorded the movement on a rotating drum.

In the second type of apparatus a specimen of *Carcinus* was free to move in a tank of aerated sea water in which a sheet of glass was suspended vertically on copper wire. The glass plate acted as a swinging partition, being large enough to prevent the specimen from passing between it and the walls of the tank. The copper wire passed through a small hole in a piece of copper plate. Whenever the crab moved the glass partition sufficiently, the wire touched the surrounding copper, completing a circuit which made a mark electro-magnetically on a smoked drum. A resistance and a condenser were used in the circuit to prevent the overlap of successive marks on the drum.

For convenience most experiments were carried out at temperatures of between 15 and 20° C. which varied by ± 1° C. over 24 hr., though some were carried out at a more accurately controlled temperature of 25° C. Illumination was constant and dim, usually from a 25 W. red lamp at a distance of 3–4 ft. Animals were not fed during the experiments.

RESULTS

Carcinus *kept moist in air*

Fig. 1 shows a typical trace by a specimen of *Carcinus* in the tipping-box aktograph during the first 24 hr. after capture. Bursts of activity occurred at times corresponding to those of high tide, and activity was greatest at the time of that high tide which occurred during what would have been the hours of darkness outside. The persistence of this rhythm of locomotory activity for a period of 3 days is illustrated in Fig. 2. Activity is plotted as the number of tilts of the box per hour. Peaks of activity occur later each day and they closely follow the succession of tides; this is particularly apparent in the exaggerated night-time peaks.

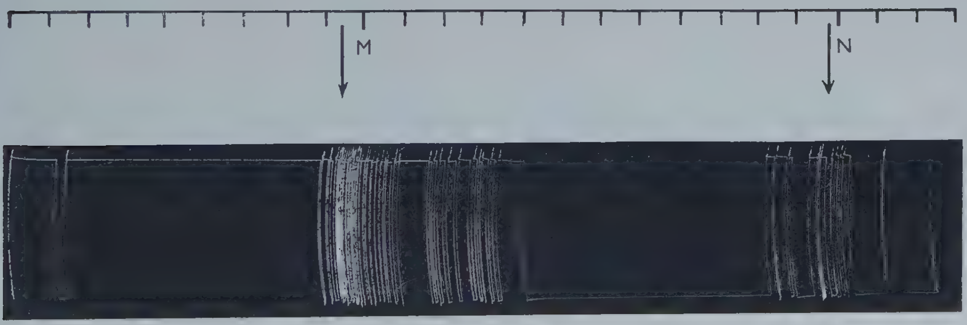

Fig. 1. Typical aktograph trace of the activity of a single *Carcinus* during the first 24 hr. after capture. (Units are hours; M is midnight, N is noon. Arrows indicate times of high tide.)

Such a rhythm persists clearly for up to 3 or 4 days in individual specimens and can be shown to be apparent for longer if the mean activity of a few crabs is considered. Fig. 3 shows the mean activity of three specimens of *Carcinus* from 21 to 23 August and of the two surviving specimens for another 4 days.

Since the activity of *Carcinus* was greater at those times when high tide would have occurred during the hours of darkness, there appeared to be a diurnal component in the rhythm. This is borne out by traces such as that in Fig. 4, which is typical of those crabs collected in summer at a time when they were experiencing both high tides during the hours of daylight. Such crabs showed a third outburst of locomotory activity during what would have been the hours of darkness, even though it would have been low tide at the time. The presence of diurnal and tidal components in the rhythm is further demonstrated in Fig. 5. This represents the summated activity of fifteen crabs, collected on successive days during a semi-lunar (15 day) cycle and each tested over a period of 48 hr. The upper histogram (Fig. 5) shows the total activity of all the crabs during each hour of the 48 hr. period irrespective of the times of high tide during each experiment. Since the experiment was over a period of 15 days the times of high tide would be evenly distributed over the whole 48 hr. period. In the lower histogram activity is plotted around the times of the four high tides which occurred during each 48 hr. period, irrespective of the time of day when they occurred. The tendency is for activity to be concentrated within 1 or 2 hr. on each side of high water and, alternatively, during and just before the hours of darkness.

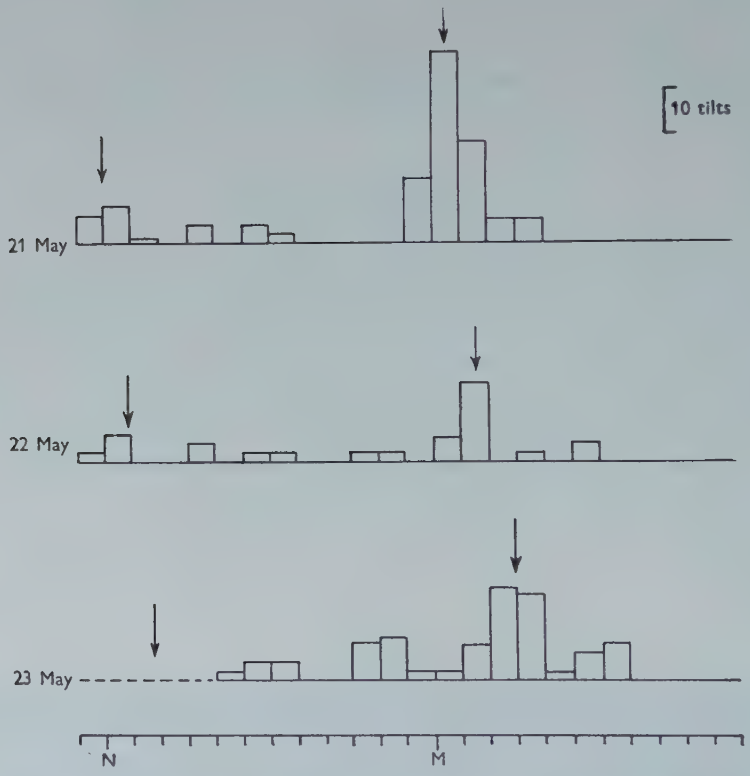

Fig. 2. Hourly activity of a single *Carcinus* from 11.00 a.m. on 21 May 1957, until 11.00 a.m. on 23 May. (Symbols as in Fig. 1; - - - -, indicates no record.)

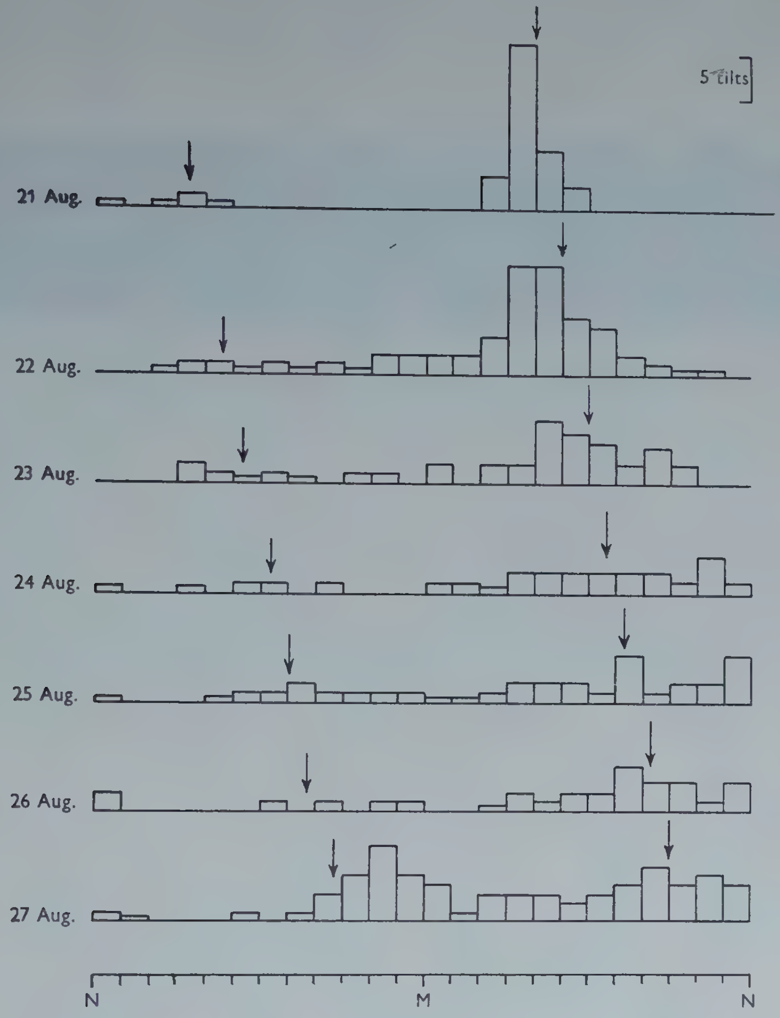

Fig. 3. Mean hourly activity of three *Carcinus* from noon on 21 August until noon on 23 August (when one specimen died), and of the two surviving *Carcinus* from noon on 23 August until noon on 27 August. (Symbols as in Fig. 1.)

Most experiments were carried out at temperatures which showed slight variations of ± 1° C. over a period of 24 hr. and it remained to determine whether such conditions were sufficiently constant. For this some of the experiments were repeated in a more accurately controlled constant temperature room. Of ten specimens tested at 25° C. all showed a persistence of the rhythm for a period of 3–4 days.

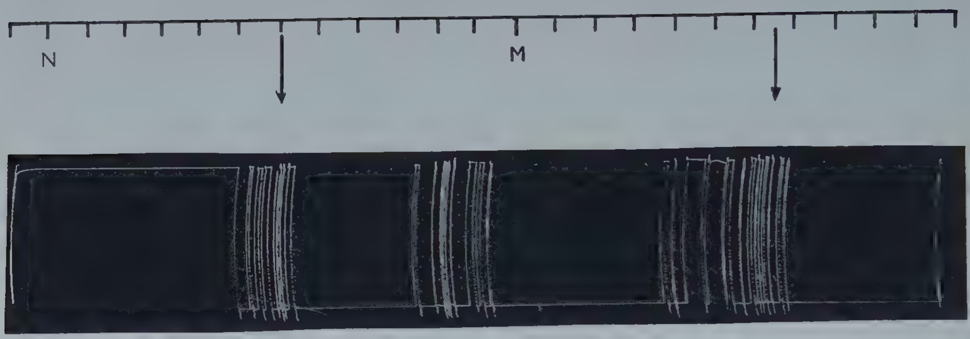

Fig. 4. Aktograph trace of a single *Carcinus* collected at a time in summer when it was experiencing both high tides during the hours of daylight. (Symbols as in Fig. 1.)

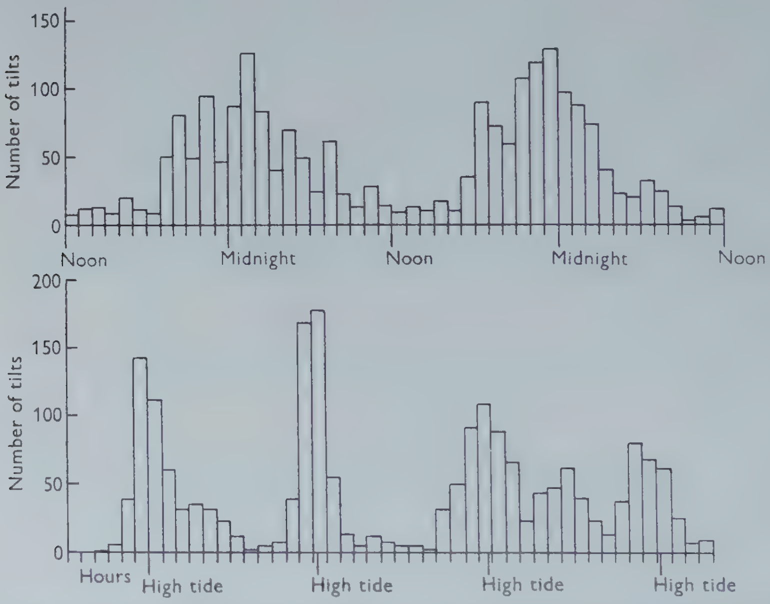

Fig. 5. Total activity of fifteen *Carcinus* collected on successive days over a semi-lunar period and each tested for 48 hr. Upper histogram shows total activity during each hour of the 48 hr. periods, irrespective of the times of high tide. Lower histogram shows total activity per hour plotted around the times of the four high tides which occurred during each 48 hr. period irrespective of the time of day when they occurred.

Carcinus *kept continuously immersed*

Experiments carried out with crabs which were continuously immersed in sea water in the second type of apparatus gave results similar to those already described. Since the apparatus is different the data obtained are comparable only qualitatively, but it is clear from Fig. 6 that both tidal and diurnal rhythms persist for 3–4 days in a crab which is continuously immersed.

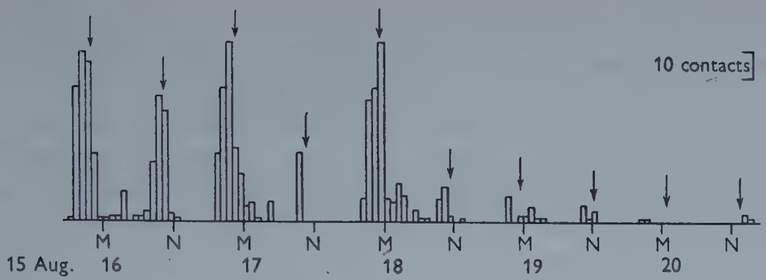

Fig. 6. Hourly activity of a single *Carcinus* kept continuously immersed in sea water from 5.00 p.m. 15 August until 5.00 p.m. 20 August. (Symbols as in Fig. 1.)

RESULTANT EFFECT OF THE TWO RHYTHMS

During June and July 1957, an extended experiment was carried out to determine the resultant effect of the two rhythms. A fresh crab was collected each day and its hourly activity was recorded during the first 24 hr. after collection. The records for all the 24 hr. periods were then plotted successively (Fig. 7). The result therefore illustrates the hourly activity of representatives of the *Carcinus* population over a period of 18 days. Early in the experiment peaks of locomotory activity were particularly high and particularly low alternately, according to whether the time of high water coincided with darkness or daylight outside. During the middle of the experiment when both high tides occurred during the hours of daylight, the peaks were moderately high and a third nocturnal peak appeared during the time of low

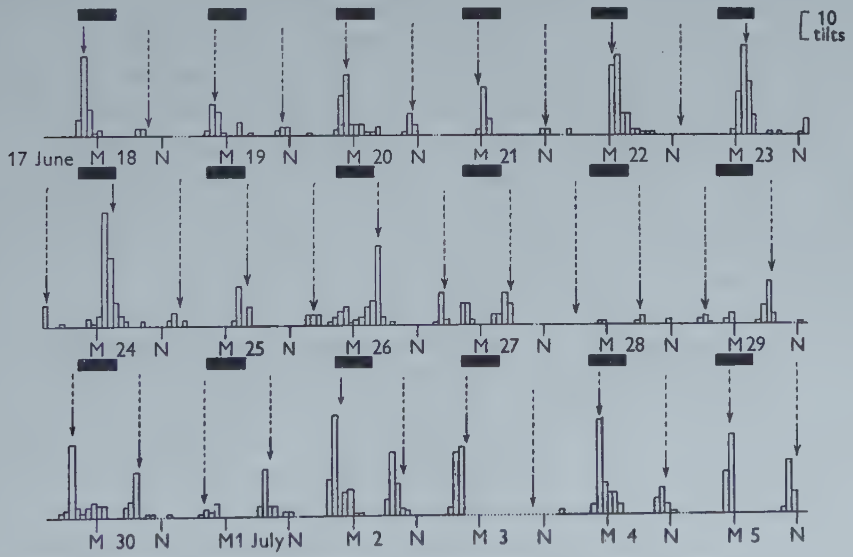

Fig. 7. Hourly activity during the first 24 hr. after capture of eighteen *Carcinus* in box-type aktographs. A specimen was freshly collected on each successive day of the experiment which ran from 2.00 p.m. 17 June 1957 until 2.00 p.m. 5 July. (Symbols as in Fig. 1; shaded rectangles indicate the hours between sunset and sunrise.)

tide. Later, when high tide again occurred in darkness, that tidal peak became particularly well marked.

This is the pattern which would be expected if two rhythms were superimposed, one of the tidal frequency (*ca.* 12·4 hr.) and one of diurnal frequency (24 hr.). Parts of the tidal cycle would become phased with the diurnal cycle every 14 or 15 days, owing to the rate of progression of the tidal cycle relative to the diurnal one. At Swansea the highest activity peaks occur during neap tides, for that is the time when high water occurs in the middle of the night.

Similar experiments carried out at Port Erin, Isle of Man, where high tides occur about 6 hr. later than at Swansea, showed similar peaks of activity, coincident with the times of high tides, and particularly well marked when high tide occurred during darkness, i.e. during spring tides in that locality. It seems probable that the diurnal and tidal cycles reinforce one another, to produce particularly well marked peaks, once a day, during periods which recur with a semi-lunar periodicity, at spring or neap tides or at any time between, depending upon locality.

DISCUSSION

The high-water peaks of activity in *Carcinus* contrast with the pattern in the fiddler crab, *Uca pugnax* (Bennett *et al.* 1957), which shows greatest activity at a time just before low tide. In both species, however, the experimental results appear to reflect the behaviour of the animals on the shore. At high tide, *Carcinus* can be seen moving freely in the littoral zone, and at low tide in daytime, they usually hide beneath stones. Nevertheless, they are sometimes found and successfully attacked by herring gulls, but these are active only during the daytime. At night *Carcinus* can safely move about on the shore during low tide. Fiddler crabs emerge from their burrows as the tide recedes and remain active until the time of low water (Bennett *et al.* 1957). The rhythms of activity in *Carcinus* and *Uca* comply with Kleitman's (1949) definition of a 'physiological rhythm' as a metabolic cycle which is synchronized with external periodicity and which persists when the external periodicity is removed. This phasing of internal rhythmicity with cyclical changes in the environment could well synchronize and facilitate the spread of the behavioural 'moods' discussed by Wells (1957).

The lack of precision of the rhythms after a few days and the diminution of activity in *Carcinus* resembles that described in *Uca* by Bennett *et al.* (1957) and in *Nassa* by Stephens *et al.* (1953). In *Uca* the broadening, warping and diminution of the peaks took place after 7 or 8 days and in *Nassa* the tidal pattern of activity persisted for 24–36 hr. at 21° C. In *Carcinus* it is unlikely that the disappearance of the rhythm resulted solely from the unusual experimental conditions in the box-type aktographs, since a similar falling off took place in crabs which were continuously immersed (Fig. 6). It may be that there are exogenous as well as endogenous components in the rhythms which have not yet been recognized, as has been suggested for *Uca* by Bennett *et al.* (1957). There is evidence for the existence of both components in some aspects of the rhythmicity of fiddler crabs (Brown, Webb

& Bennett, 1955; Brown, Webb, Bennett & Sandeen, 1955). It might of course be of advantage to littoral animals if their rhythmicity was not so well implanted as, for instance, are some diurnal rhythms in fresh-water species (Harker, 1953) and terrestrial species (Cloudsley-Thompson, 1951). Some mayfly nymphs show a reaction to the light-darkness rhythm which may be 'in some measure inherent' (Harker, 1953), and inherited rhythms also occur in *Drosophila* (see Brown, 1957). Since many activity rhythms in shore animals are phased by local tides and since the times of high tide may vary over relatively short distances it might be disadvantageous if an existing rhythm could not be readily modified to suit a new tidal regime. Adult animals might not normally be widely transported, but in those species such as *Carcinus* which have a planktonic dispersal stage selection would no doubt be against an inherent tidal rhythm which could not be modified. Rao (1954) has shown that *Mytilus* transported from Barnstaple Harbour, Cape Cod, to Los Angeles, became adapted to the new tidal cycle after only 7 days. Brown (1954) has gone further than this with oysters. When these were transported to a new locality and maintained in non-tidal conditions, they showed for the first few days a statistical rhythm in phase with tides of the native habitat, but after about 14 days they had reverted to a rhythm which showed maxima at the times of lunar zenith and lunar nadir. Such a rhythm is of a frequency which could readily be phased with the tidal cycle in almost any locality.

A final point which is possibly of wider significance arises from the demonstration of two rhythms of different frequencies in marine animals. In addition to the locomotory rhythms in *Carcinus* diurnal and tidal rhythms of various kinds have been described in *Uca pugnax* and *U. pugilator* (Brown *et al.* 1953; Brown *et al.* 1954; Brown *et al.* 1955), *Ostrea virginica* (Brown, 1954), *Venus mercenaria* (Bennett, 1954), *Littorina littorea* and *Urosalpinx cinereus* (Sandeen *et al.* 1954), and *Callinectes sapidus* (Fingerman, 1955). The resultant pattern of such rhythms is of semi-lunar frequency and it might well be a factor regulating periodic spawning behaviour (Brown *et al.* 1953, 1954, 1955; Brown, 1954; Bennett, 1954) in addition to those which have previously been considered by Korringa (1947), Loosanoff & Nomejko (1951), Knight-Jones (1952) and Fox (1956).

SUMMARY

1. Spontaneous locomotory activity was recorded continuously for several days in *Carcinus maenas* (L.).

2. A complex rhythm was observed which could be analysed into two components, one of diurnal frequency (24 hr.), with peaks of activity during the hours of darkness, and one of tidal frequency (*ca.* 12·4 hr.), with peaks at the time of high tide.

3. The rhythms persist in constant dim light at constant temperatures, whether the crabs are kept moist in air or continuously immersed in sea water.

4. The resultant effect of the two rhythms is to produce particularly high activity peaks during periods which recur with a semi-lunar frequency.

5. Experimental results relate to observed behaviour on the shore.

I am indebted to Prof. E. W. Knight-Jones for help and advice given throughout the course of this work. My thanks are also due to Mr A. Macfadyen for helpful discussion and to Dr K. A. Munday for advice about the second type of apparatus. Mr J. S. Colman kindly provided laboratory facilities for experiments carried out at the Marine Biological Station, Port Erin.

REFERENCES

BENNETT, M. F. (1954). The rhythmic activity of the quahog, *Venus mercenaria*, and its modification by light. *Biol. Bull., Woods Hole*, **107**, 174–91.

BENNETT, M. F., SHRINER, J. & BROWN, R. A. (1957). Persistent tidal cycles of spontaneous motor activity in the fiddler crab, *Uca pugnax*. *Biol. Bull., Woods Hole*, **112**, 267–75.

BOHN, G. (1906). La persistance du rhythme des marées chez l'*Actinia equina*. *C.R. Soc. Biol. Paris*, **61**, 661–3.

BROWN, F. A., JR., FINGERMAN, M., SANDEEN, M. I. & WEBB, H. M. (1953). Persistent diurnal and tidal rhythms of color change in the fiddler crab, *Uca pugnax*. *J. Exp. Zool.* **123**, 29–60.

BROWN, F. A., JR. (1954). Persistent activity rhythms in the oyster. *Amer. J. Physiol.* **178**, 510–14.

BROWN, F. A., BENNETT, M. F. & WEBB, H. M. (1954). Persistent daily and tidal rhythms of O_2-consumption in fiddler crabs. *J. Cell. Comp. Physiol.* **44**, 477–505.

BROWN, F. A., JR., WEBB, H. M. & BENNETT, M. F. (1955). Proof for an endogenous component in persistent solar and lunar rhythmicity in organisms. *Proc. Nat. Acad. Sci., Wash.*, **41**, 93–100.

BROWN, F. A., JR., WEBB, H. M., BENNETT, M. F. & SANDEEN, M. I. (1955). Evidence for an exogenous contribution to persistent diurnal and lunar rhythmicity under so-called constant conditions. *Biol. Bull., Woods Hole*, **109**, 238–54.

BROWN, F. A., JR. (1957). The rhythmic nature of life. *Recent Advances in Invertebrate Physiology*, ed. B. T. Scheer, pp. 287–304. University of Oregon.

CLOUDSLEY-THOMPSON, J. M. (1951). Studies in diurnal rhythms. I. Rhythmic behaviour in millipedes. *J. Exp. Biol.* **28**, 165–72.

FINGERMAN, M. (1955). Persistent daily and tidal rhythms of color change in *Callinectes sapidus*. *Biol. Bull., Woods Hole*, **109**, 255–64.

FOX, H. M. (1956). The moon and life. *Proc. Roy. Instn G.B.* **37**, (163), 1–12.

GAMBLE, F. W. & KEEBLE, F. (1903). The bionomics of *Convoluta roscoffensis* with special references to its green cells. *Proc. Roy. Soc.* B, **72**, 93–8.

GAMBLE, F. W. & KEEBLE, F. (1904). The bionomics of *Convoluta roscoffensis* with special reference to its green cells. *Quart. J. Micr. Sci.* **47**, 363–431.

GOMPEL, M. (1937). Recherches sur la consommation d'oxygène de quelques animaux aquatiques littoraux. *C.R. Acad. Sci., Paris*, **205**, 816–18.

HARKER, J. E. (1953). The diurnal rhythm of activity of mayfly nymphs. *J. Exp. Biol.* **30**, 525–33.

KLEITMAN, N. (1949). Biological rhythms and cycles. *Physiol. Rev.* **29**, 1–30.

KNIGHT-JONES, E. W. (1952). Reproduction of oysters in the Rivers Crouch and Roach, Essex, during 1947, 1948 and 1949. *Fish. Invest., Lond.*, Ser. II, **18** (2), 1–48.

KORRINGA, P. (1947). Relations between the moon and periodicity in the breeding of marine animals. *Ecol. Monogr.* **17**, 347–81.

LOOSANOFF, V. L. & NOMEJKO, C. A. (1951). Spawning and setting of the American oyster, *O. virginica*, in relation to lunar phases. *Ecology*, **32**, 113–34.

RAO, K. P. (1954). Tidal rhythmicity in the rate of water propulsion in *Mytilus*, and its modifiability by transplantation. *Biol. Bull., Woods Hole*, **106**, 353–9.

SANDEEN, M. I., STEPHENS, G. C. & BROWN, F. A., JR. (1954). Persistent daily and tidal rhythms of oxygen consumption in two species of marine snails. *Physiol. Zoöl.* **27**, 350–6.

STEPHENS, G. C., SANDEEN, M. I. & WEBB, H. M. (1953). A persistent tidal rhythm of activity in the mud snail, *Nassa obsoleta*. *Anat. Rec.* **117**, 635.

WELLS, G. P. (1957). The sources of animal behaviour. *Smithsonian Rep. for* 1956, 415–29.

THE ABSORPTION AND METABOLISM OF SOME SUGARS IN THE LOCUST, *SCHISTOCERCA GREGARIA* (FORSK.)

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INTRODUCTION

The absorption of labelled glucose from the mid-gut of *Schistocerca gregaria* (Forsk.) has been shown to be related to the rate of its conversion to trehalose, which accumulates in the haemolymph (Treherne, 1958*a*). The rate of disappearance of glucose from the isolated alimentary canal suspended in a relatively large volume of circulating poisoned saline was similar to the absorption obtained in the intact animal when there was a rapid conversion to trehalose. On the basis of these observations it was suggested that the mechanism of glucose absorption might involve a diffusion across the gut wall, this process being facilitated by the rapid conversion to trehalose which would tend to maintain a steep concentration gradient. The similarity of this hypothesis to the early, and now outmoded, theories of Höber (1899) and Verzár (1931) for the absorption of hexoses from the mammalian intestine was noted (Treherne, 1958*a*, *b*).

In the present investigation experimental observations have been extended to mannose and fructose in an attempt to throw some further light on the nature of the absorptive processes involved. A detailed analysis has also been made of the absorption of labelled glucose at low concentration, for the uptake at these levels is of considerable theoretical interest in interpreting the extent of the facilitated diffusion and the role of any other absorptive processes in the absorption of sugars by this insect.

METHODS

Many of the techniques employed in this investigation were similar to those used in previous studies (Treherne, 1957, 1958*a*) and for this reason detailed descriptions have been limited to techniques incorporating novel features.

The experiments were performed on adult female *S. gregaria* (Forsk.) which were reared and maintained at 28·0 ± 1·0° C. and fed on fresh green wheat grown in pots. To empty the alimentary canal individuals were isolated in glass jars and deprived of food for 24 hr. preceding the experiment.

In order to fill the alimentary canal with the experimental solution a fine nylon hypodermic needle was thrust into the rectum of an insect anaesthetized with CO_2, and 0·15 ml. of fluid forced into the lumen of the gut. By this method the whole of the hind-gut and mid-gut was filled with the experimental solution.

The amount of ^{14}C-labelled monosaccharide absorbed from each part of the gut was determined using a dye, Amaranth (Azo-Rubin S), as a marker. This dye was not absorbed from the lumen of the gut and the percentage absorption of the labelled sugars was calculated from the glucose/dye ratio in the various parts of the alimentary canal. Amaranth has also been found suitable for use as a blood volume indicator by Yeager & Munson (1950). The dye concentration used in this investigation was 0·05 M/l. The experimental solution used was based on the saline described by Hoyle (1953). When the concentration of the sugars was altered, in the range 0·00134–0·30 M/l., the total osmolarity of the solution was maintained by appropriate adjustment of the NaCl concentration.

The ^{14}C-labelled sugars used in this investigation were glucose, fructose and mannose obtained from the Radiochemical Centre, Amersham. In each case the radio-active molecules were generally labelled with ^{14}C. The radio-activity was assayed in solution as previously described (Treherne, 1957) or counted on lens paper (Reid, 1947), using a thin-windowed G.M. tube (G.E.C. CV 2139).

Initially the experimental solution was collected from the gut lumen by removing the gut and dropping the washed ligatured portions into known volumes of a solution buffered to pH 10·0. In some later experiments it was found necessary to obtain samples of the contents of the mid-gut caeca in which there was no trace of contamination by the haemolymph. For this purpose 0·5 mm. diameter nylon tube was drawn out over a low flame and a 7·0 cm. length was fixed to an ordinary hypodermic needle-holder mounted on to a 1·0 ml. syringe. The dead space of the syringe and nylon tube was filled with liquid paraffin. The locust was anaesthetized with CO_2 and quickly opened to expose the alimentary canal. The nylon tube was then thrust into the rectum and pushed gently forward until the tip was in the region of the caeca. A small volume of fluid was then withdrawn into the tube by suction and the tube removed from the gut.

The separation and identification of carbohydrates from the haemolymph was accomplished by descending paper chromatography using Whatman No. 1 filter-paper. The following solvent systems were employed: ethyl acetate/acetic acid/water (Jermyn & Isherwood, 1949), *n*-butanol/ethanol/water (Hirst & Jones, 1949) and *n*-propanol/ethyl acetate/water (Baar & Bull, 1953). Reducing substances on the chromatograms were detected using the silver-nitrate method of Trevelyan, Proctor & Harrison (1950). The presence of non-reducing substances was revealed by spraying with 0·5 % sodium-*meta*periodate (Evans & Dethier, 1957) before treatment with silver nitrate. Control experiments showed that single spots of haemolymph applied to the base line of the chromatograms did not interfere with any of the solvent systems used. When more than one spot was applied to the same portion of the paper then some interference resulted. It was therefore not possible to concentrate the haemolymph at the base line by successive applications of haemolymph to one portion of the paper. It was found, however, that if the haemolymph was coagulated by heating to 100° C. and then centrifuged the resulting supernatant did not interfere and the carbohydrate could be safely concentrated on the chromatogram by successive applications of this fluid.

To determine the concentration of glucose and trehalose in the haemolymph these substances were eluted from the chromatograms and determined by the anthrone method described by Dimler, Schaefer, Wise & Rist (1952). Glucose was found to be present in relatively small amounts and it was found necessary to apply at least 25 vol. of a 3·0 μl. pipette in a row along the base line of the chromatogram.

Blood volume determinations were carried out by the dye method of Yeager & Munson (1950).

RESULTS

The uptake of labelled mannose and fructose from the various parts of the alimentary canal during an experimental period of 15·0 min. was studied at concentrations of 0·002, 0·02 and 0·20 M/l. At all three concentrations the uptake was found to be confined to the caeca and ventriculus (Figs. 1, 2). The disappearance of mannose from the caeca was found to proceed more rapidly at a concentration of 0·002 M/l. than at 0·02 M/l., a difference which was statistically significant ($P < 0·01$). There was a smaller difference between the percentage absorption at 0·02 and 0·20 M/l., which was nevertheless significant ($P \simeq 0·02$). The disappearance of labelled fructose (Fig. 2) was similar at all three concentrations, except for the barely significant difference ($P \simeq 0·05$) between the absorption from the ventriculus at 0·02 and 0·20 M/l. The results for the absorption of mannose and fructose from the caeca are summarized and compared with those for glucose (Treherne, 1958*a*) in Table 1. It will be seen that the percentage absorption of glucose was relatively high at 0·002 and 0·02 M/l., but was significantly less at 0·20 M/l. Mannose showed a progressive decline from a rate which was similar to that of glucose at 0·002 M/l., while fructose showed a relatively slow absorption at all three concentrations.

To follow the disappearance of mannose and fructose from the gut lumen *in vitro* the alimentary canal was removed from an anaesthetized insect and filled via the rectum with 0·15 ml. of 0·02 M/l. sugar solution. The alimentary canal was then ligatured at the crop and the rectum and suspended in 1·0 l. of saline which was kept circulating around the gut by a stream of air bubbles. The circulating saline contained KCN and iodoacetic acid each to a concentration of 2·0 mM/l. With both mannose and fructose it was found that the absorption from the caeca in the isolated poisoned alimentary canal was more rapid than in the intact insect (Fig. 3). These differences were found to be statistically significant ($P < 0·01$ in both cases). The uptake of these sugars *in vitro* was similar to that for glucose where the percentage absorption from the caeca under identical experimental conditions was 54·5 ± 6·0% (Treherne, 1958*a*).

Experiments were carried out which were designed to investigate the relation between the monosaccharides in the gut lumen and the carbohydrates in the haemolymph. To do this solutions containing radio-active sugars were injected into the gut lumen and the ^{14}C-labelled compounds found in the haemolymph were examined by paper chromatography. Fig. 4 illustrates the distribution of radio-activity in the haemolymph for the three sugars. In this experiment the initial concentration of the sugars in the gut lumen was 0·02 M/l., the experimental period being 15 min.

It will be seen that in all three cases a peak of radio-activity was found which coincided with that for trehalose. Under these conditions all of the labelled glucose was converted to trehalose, but with both the other sugars substantial amounts of mannose and fructose remained in the haemolymph. The relation between the sugar concentration in the gut lumen and the amount of trehalose formed in the haemolymph is illustrated in Fig. 5. With glucose there was a complete conversion to trehalose until a concentration of just over 0·02 M/l. was reached in the lumen, after which there was a rapid fall in the percentage of trehalose formed in the haemolymph.

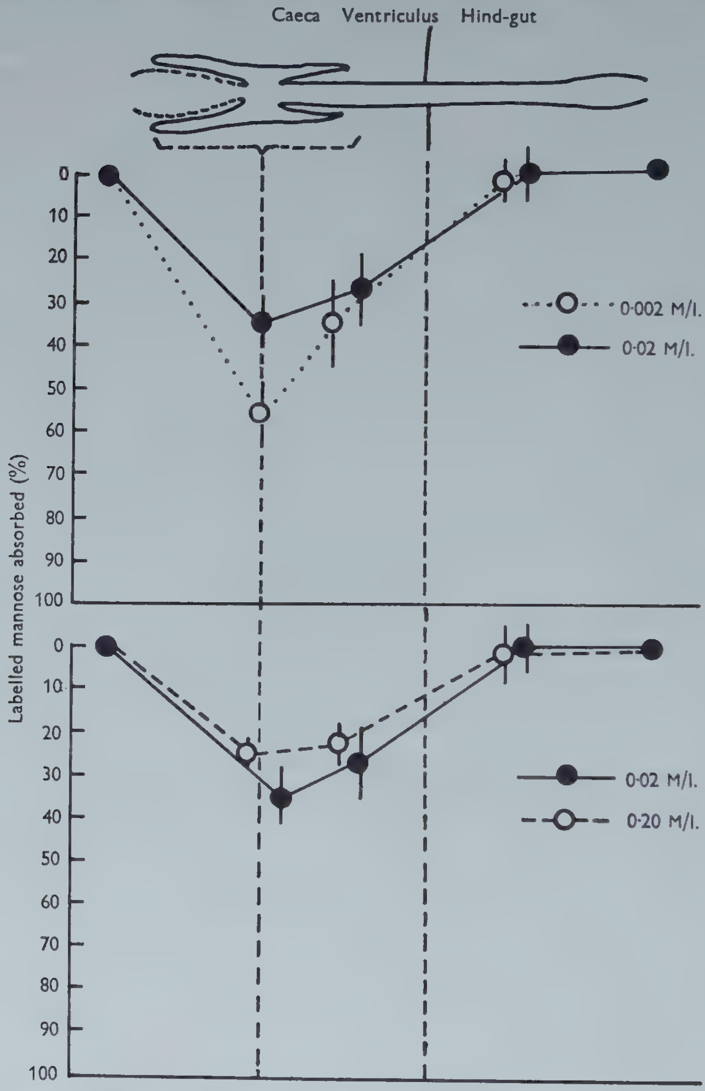

Fig. 1. The uptake of ^{14}C-labelled mannose from the alimentary canal at three concentrations. Each symbol represents the mean and extent of the standard deviation for five determinations.

With mannose there was a complete conversion at low concentration, but the amount of trehalose formed fell off much more rapidly with increasing concentration than with glucose. With fructose there was only a relatively slow conversion to trehalose at all the concentrations studied.

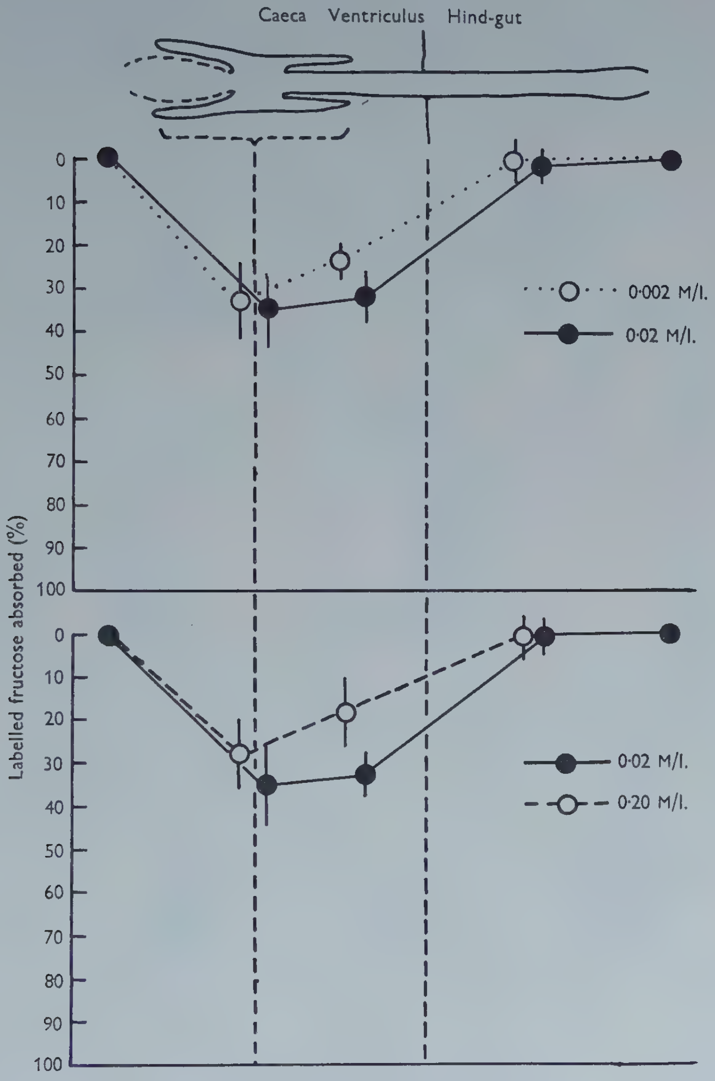

Fig. 2. The uptake of ^{14}C-labelled fructose from the alimentary canal at three concentrations. Each symbol represents the mean and extent of the standard deviation for five determinations.

In some experiments the extent of the conversion of labelled sugars absorbed by the mid-gut was compared with that obtained on direct injection of sugars into the haemolymph. With glucose, for example, it was estimated that with a concentration in the gut lumen of 0·02 M/l. a total of 0·138 mg. of labelled glucose was absorbed

Table 1. *The percentage uptake of ^{14}C-labelled sugars from the mid-gut caeca at three concentrations during an experimental period of 15 min.*

Sugar	Labelled sugar absorbed (%)		
	0·002 M/l.	0·02 M/l.	0·20 M/l.
Glucose	54·1 ± 6·7	55·7 ± 7·9	39·1 ± 6·1
Mannose	55·2 ± 7·2	35·5 ± 6·8	26·3 ± 1·5
Fructose	32·3 ± 9·5	34·8 ± 8·9	27·5 ± 8·1

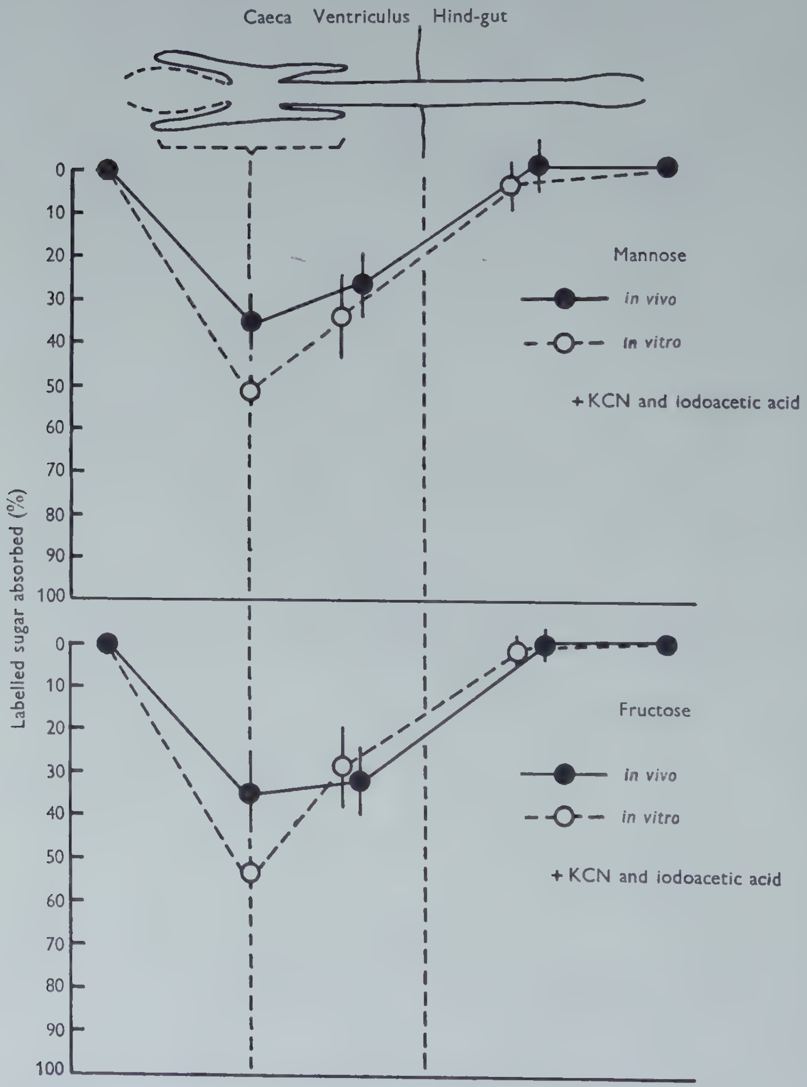

Fig. 3. The percentage absorption of labelled mannose and fructose from a poisoned gut isolated in circulating saline compared with that from the intact animal, during an experimental period of 15·0 min. The various symbols represent the mean and extent of the standard deviation.

from the caeca and the ventriculus during the experimental period of 15·0 min. Therefore in this experiment 0·138 mg. of ^{14}C-labelled glucose was injected into the haemolymph in 0·03 ml. saline using an Agla syringe. The results of these

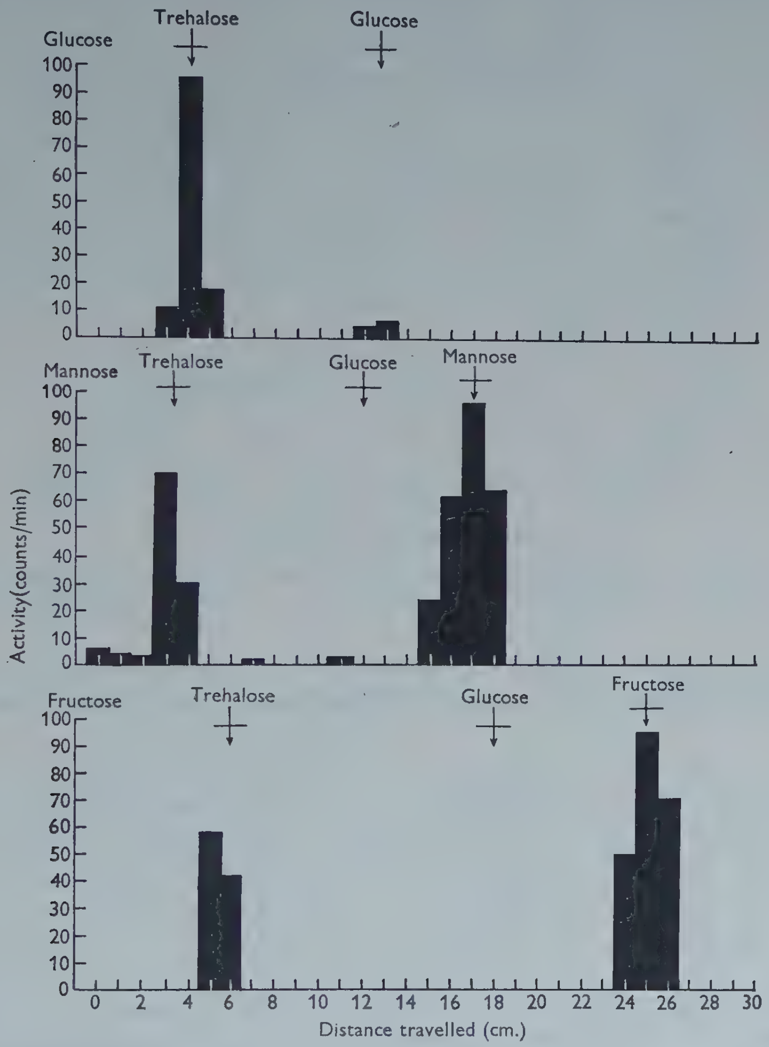

Fig. 4. The radio-activity on paper chromatograms of haemolymph taken 15·0 min. after the introduction of ^{14}C-labelled sugars into the alimentary canal. The symbols above the chromatograms represent the position and extent of the control spots, which were run adjacent to the experimental ones. The solvent system used in these experiments was ethyl acetate/acetic acid/water.

experiments are summarized in Table 2. It seems fairly clear that the percentage conversion to trehalose was approximately the same whether the sugars were absorbed via the gut or injected directly into the haemolymph.

The concentration of glucose and trehalose in the haemolymph was measured by eluting these sugars from the paper chromatograms and determining their concentration by the anthrone method (Dimler *et al.* 1952). The results are given in Table 3. It will be seen that trehalose was present in fairly massive amounts. There was a considerable degree of variation in the results for glucose, but it is clear that this sugar formed only a small proportion of the total (about 3·4 %). These findings in general are similar to those published by Howden & Kilby (1956) for this species, although the figures for glucose are somewhat lower than the values of 50–100 mg. % quoted by these authors.

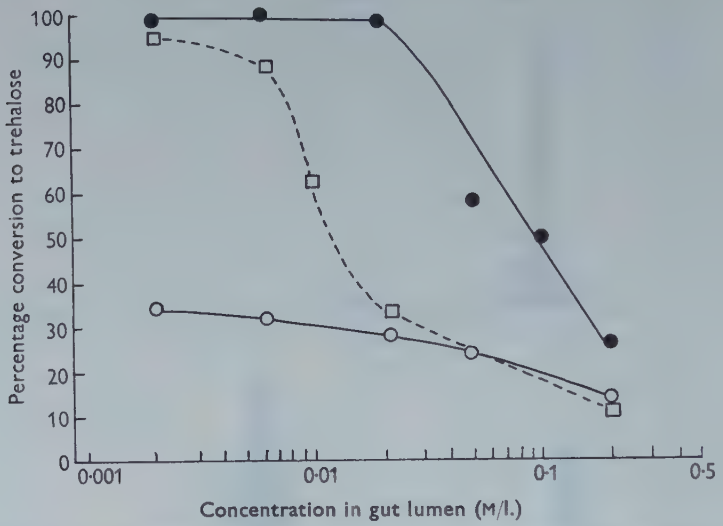

Fig. 5. The effect of concentration of ^{14}C-labelled sugars in the gut lumen on the amount of trehalose found in the haemolymph. —●—, glucose; - - □ - -, mannose; —○—, fructose.

Table 2. *A comparison of the rates of conversion to trehalose of ^{14}C-labelled sugars when absorbed via the mid-gut or injected directly into the haemolymph*

Sugar	Concentration in gut lumen	% incorporated as trehalose in the haemolymph	
		Absorbed via the mid-gut	Injected into the haemolymph
Glucose	0·002 M/l.	98·6 ± 2·3	97·9 ± 1·8
	0·02	98·6 ± 6·5	91·9 ± 7·5
Fructose	0·02 M/l.	28·3 ± 3·9	34·3 ± 4·5

The results in Table 3 indicate that at the lowest concentrations used in this investigation the concentration of glucose in the gut lumen approached that in the haemolymph. At these concentrations in the lumen the uptake of labelled glucose could be due either to an active transport or to passive exchange with the glucose in equilibrium with the trehalose in the haemolymph. In order to differentiate between these two possibilities it is essential to be able to measure the net glucose absorption at very low concentrations in the lumen of the mid-gut. If much of the labelled glucose was being actively absorbed then it should be possible to demonstrate a substantial net glucose absorption; if the labelled glucose was merely exchanging with that in the haemolymph then there would be no demonstrable net glucose absorption. Unfortunately, it was not possible to measure directly the net glucose uptake at these concentrations because of the difficulty of making accurate determinations by chemical analysis of such small quantities of sugar. An attempt was made, therefore, to estimate the net absorption at low concentration by investigating the uptake of labelled glucose from the mid-gut caeca when the initial specific activity of the glucose in the haemolymph approximated to that in the gut lumen. This was done by injecting sufficient radio-active glucose solution into the haemolymph to give the same specific activity on both sides of the gut wall. Under these conditions any decrease in the labelled glucose in the lumen will be a measure of the net glucose absorption. These results were compared with those in which the labelled glucose was absorbed, as in previous experiments, into the haemolymph containing no ^{14}C. Because of the rapidity with which the glucose exchanges and becomes incorporated as trehalose in the haemolymph, sufficient radio-active glucose was injected so that the specific activity of the glucose and the trehalose equalled that of the glucose in the gut lumen. To take an actual example, the blood volume of this insect was found to be 0·248 ± 0·038 ml., so that from the results in Table 3 it can be estimated that the total amount of glucose and trehalose (expressed as glucose units) was approximately 3·50 mg. Now the specific activity of a 0·00134 M/l. glucose solution in the gut lumen was 0·655 μc./mg., therefore it was necessary to inject 2·29 μc. of ^{14}C-labelled glucose into the haemolymph to bring the glucose in the haemolymph to the same specific activity as the glucose in the gut contents. The labelled glucose was allowed to come into equilibrium with trehalose for a period of 30·0 min. before the gut lumen was filled with the experimental solution. It was assumed that after this period the small amount of glucose in equilibrium with the trehalose in the haemolymph was of a similar specific activity to that in the gut lumen. The labelled glucose was injected into the haemolymph in a small

Table 3. *The concentration of glucose and trehalose in the haemolymph of adult female locusts*

Serial	Glucose concentration mg./100 ml.	Trehalose concentration mg./100 ml.
1	32·2	795·9
2	12·3	801·6
3	23·5	545·7
4	34·7	622·3
5	9·8	674·2
6	6·3	599·5
7	40·1	847·6
8	34·2	669·0
Mean ± S.D.	24·1 ± 12·9	694·5 ± 99·3

volume of saline with the aid of an Agla syringe. To minimize the effects of contamination of the gut contents by the haemolymph the fluid in the caeca was collected by means of the nylon tubing previously described. The results obtained

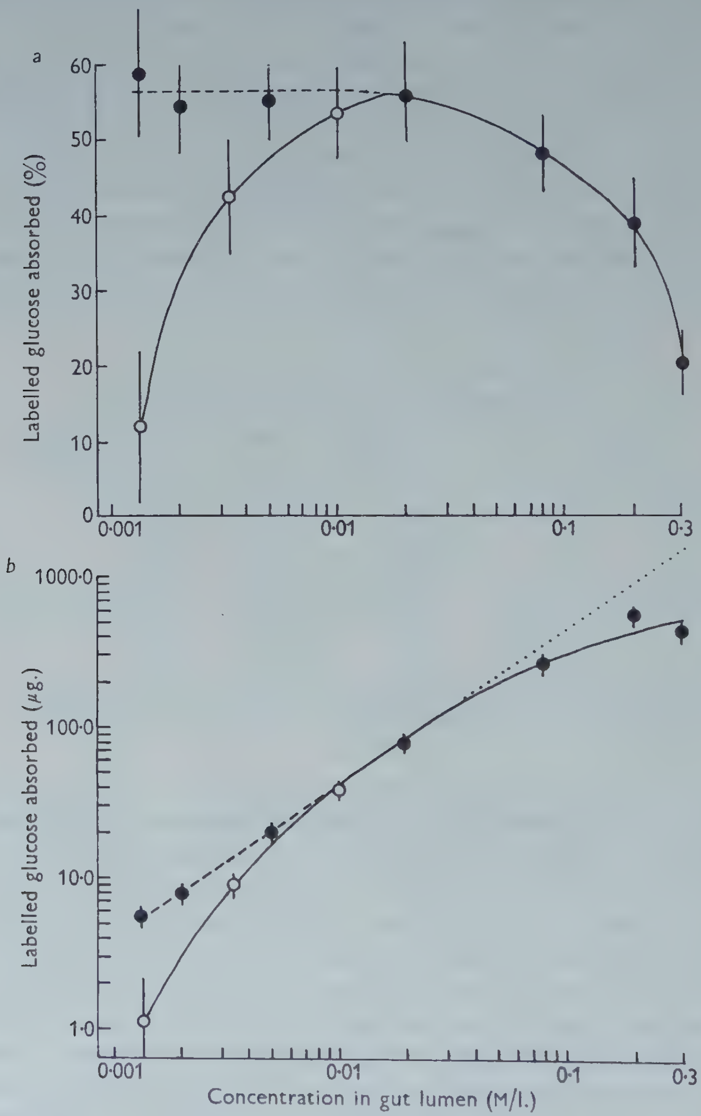

Fig. 6. (*a*) The effect of concentration on the movements of ^{14}C-labelled glucose between the lumen of the caeca and the haemolymph. The open circles represent the percentage absorption of labelled glucose when the initial radio-activity of the glucose in the haemolymph, in equilibrium with the trehalose, approximated to that in the gut lumen. The closed circles represent the uptake when there was no initial radio-activity in the haemolymph. The vertical lines illustrate the extent of the standard deviation. (*b*) A logarithmic plot illustrating the uptake of labelled glucose, in absolute units, calculated from the data illustrated in (*a*). The continuous line represents the net glucose absorption, while the broken one indicates the net absorption plus the apparent absorption due to exchange with unlabelled molecules.

using this technique are illustrated in Fig. 6*a*. It will be seen that at a concentration of 0·00134 M/l. the percentage absorption of labelled glucose from the caeca was much reduced in the experiments in which the specific activity in the haemolymph was similar to that in the gut lumen. This difference became smaller as the glucose concentration of the gut contents was increased, so that at 0·01 M/l. the percentage absorption of the labelled glucose was similar in both cases. In Fig. 6*b* the absolute amount of labelled glucose (in μg.) absorbed from the lumen of the caeca during the experimental period has been plotted on a logarithmic scale with respect to concentration. These figures were calculated from the data given in Fig. 6*a* and with the knowledge that the initial volume of the caeca contents was 0·039 ± 0·009 ml. In both graphs the continuous line can be regarded as an estimate of the net glucose absorption effected during the experimental period.

It seemed possible that the apparent reduction in the percentage absorption of labelled glucose in the caeca at low concentrations, when the specific activity of the haemolymph equalled that in the gut lumen, might be due, in part at least, to an entry of radio-active material into the lumen of the gut before the introduction of the experimental solution. The further possibility could not be eliminated that, despite careful precautions, there might be some contamination of the caeca contents by the highly radio-active haemolymph due to slight damage to the gut wall during collection of the caecal fluid. These possibilities were tested in the following way. 2·92 μc. ^{14}C-labelled glucose were injected into the haemolymph. The experimental fluid from the caeca was then immediately collected and the amount of radio-active material present determined in relation to the recovered dye. If any extraneous radio-active material had entered the lumen of the caeca during the equilibration period this would have been reflected in an increase in the amount of radio-activity relative to the recovered dye. If no radio-active material had entered the lumen then the glucose/dye ratio would not have altered and the apparent percentage absorption would have been zero. The apparent absorption in these experiments was $-0·80 \pm 3·80$%, indicating that the entry of any extraneous material into the gut lumen during the equilibration period was relatively small.

DISCUSSION

The absorption of the labelled sugars was found to be largely confined to the mid-gut, a finding which is in accord with some previous investigations on glucose absorption in the cockroach (Treherne, 1957) and the locust (Treherne, 1958*a*). The proportion of the labelled sugars absorbed from the lumen of the caeca, however, showed some marked differences at different concentrations. The uptake of labelled glucose was relatively rapid at 0·002 and 0·02 M/l., but fell to a rate which was similar to that from the ventriculus at 0·20 M/l. With mannose the percentage absorption was similar to that for glucose at 0·002 M/l. but then declined much more rapidly, again to a rate similar to that from the ventriculus. Fructose showed a rather slow rate of absorption at all three concentrations.

The absorbed sugars were converted, in varying degrees, to trehalose which accumulated in the haemolymph. This non-reducing disaccharide was first detected in insects by Wyatt & Kalf (1956) and its presence in *S. gregaria* was confirmed by Howden & Kilby (1956). It was recently shown that absorbed glucose was incorporated as trehalose in this insect (Treherne, 1958*a*). The conversion to trehalose of the three sugars used in this investigation showed considerable differences and variations when the concentration in the gut lumen was altered. In these experiments glucose showed a very rapid conversion to trehalose, until the concentration in the gut lumen exceeded 0·02 M/l. when excess glucose accumulated in the haemolymph. Mannose was rapidly converted to trehalose at a concentration of 0·002 M/l. in the gut lumen, but the rate of conversion fell off steeply with increasing concentration. With fructose the conversion proceeded relatively slowly at all concentrations studied. It seems clear that these rates of conversion to trehalose parallel the percentage absorption of the labelled sugars from the lumen of the caeca. These facts do not conflict with the hypothesis advanced in a previous publication (Treherne, 1958*a*) that glucose absorption is effected by diffusion across the gut wall and that the process is facilitated by the conversion to trehalose which would tend to maintain a steep concentration gradient across the gut wall. Evans & Dethier (1957) have reported changes in the blood sugar concentration in the blowfly which suggest a fairly rapid conversion of mannose and fructose to trehalose and it is possible that in this species sugar absorption may be similar to that in the locust.

When the alimentary canal was isolated in circulating poisoned saline the three sugars disappeared from the gut lumen at similar rates. The passage of glucose through the gut wall under these circumstances was at the same rate as in the intact animal when there was a complete conversion to trehalose. With mannose and fructose this disappearance from the gut lumen *in vitro* was more rapid than in the intact animal, where at 0·02 M/l. the conversion to trehalose was relatively slow. The behaviour of the sugars in this model system lends support to the hypothesis of absorption by facilitated diffusion, for in these circumstances it would be expected that the sugars would diffuse through the gut wall at similar rates. The slower absorption of mannose and fructose from the intact gut, as compared with that *in vitro*, can be attributed to the reduced concentration gradient across the gut wall due to the demonstrated accumulation of unconverted monosaccharides in the haemolymph at this concentration.

It was found that the conversion to trehalose occurred at closely similar rates whether the sugars were absorbed via the mid-gut or injected directly into the haemolymph. This suggests that the postulated absorption mechanism could operate by converting the absorbed sugars in the haemolymph, thus maintaining a concentration gradient across the whole width of the gut wall. These results do not, however, eliminate the possibility of conversion occurring within the mucosal cells.

Chemical analysis of the haemolymph showed that there was a small amount of glucose in equilibrium with the trehalose, the values obtained for glucose being somewhat smaller than those quoted by Howden & Kilby (1956). Thus at very low concentrations the glucose in the gut lumen was at a similar level to that in the haemolymph (i.e. 0·00134 M/l.). The uptake of labelled glucose from the caeca at low concentrations was shown to be substantially reduced when the initial specific activity of the glucose in the haemolymph was adjusted to approximate to that in the gut lumen. This effect became negligibly small when a concentration of about 0·01 M/l. was attained in the gut lumen. Under these experimental conditions the absorption of the labelled glucose can be regarded as an estimate of the *net* glucose absorption effected from the mid-gut caeca. It seems fairly clear that at very low concentrations, close to that in the haemolymph, most of the absorption of labelled glucose from the caeca was due merely to exchange with that in the haemolymph. With increasing concentration in the lumen the proportion exchanging with that in the haemolymph became progressively smaller and the absorption of the labelled glucose molecules approximated to the net glucose absorption. The exchange and absorption of glucose between the gut lumen and in the haemolymph are illustrated diagrammatically in Fig. 7. There was no evidence for any appreciable amounts of mannose or fructose in the haemolymph; therefore in experiments with these sugars

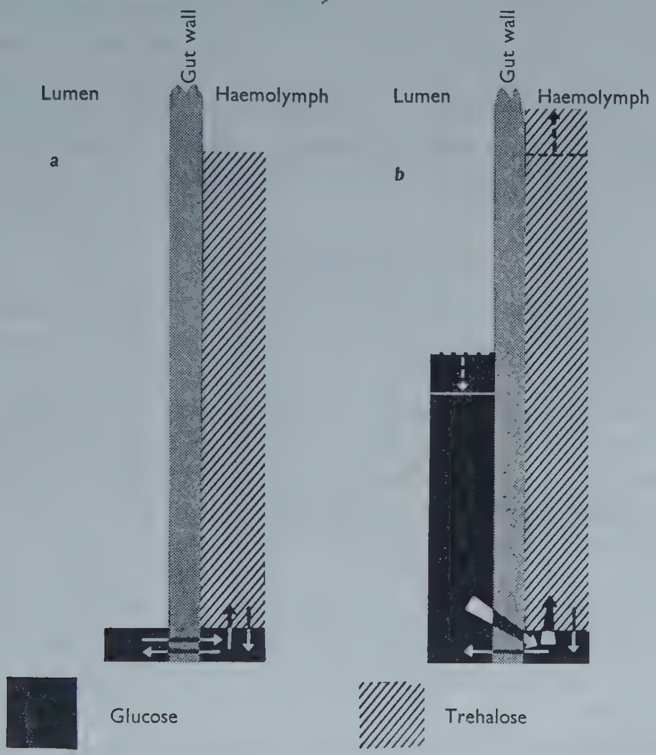

Fig. 7. A diagrammatic representation of the movements of labelled glucose between the lumen of the caeca and in the haemolymph: (*a*) At low concentration, when the glucose level in the lumen is similar to that in the haemolymph. (*b*) At a relatively high concentration when the glucose in the lumen exceeds that in the haemolymph. (Not drawn to scale.)

the uptake of labelled molecules will represent fairly closely the net absorption effected by the mid-gut.

It will be appreciated that the conversion to trehalose could only produce a net glucose uptake when the concentration in the gut lumen exceeded that in the haemolymph. At very low concentrations most of the glucose movements would be an exchange with that in the haemolymph (Fig. 7*a*). Therefore, it is clear that at low concentrations, such as might exist towards the end of a meal, some additional mechanism must be postulated to explain any net glucose absorption observed. The experiments showed that the net uptake probably proceeded very slowly at low concentration, and it follows that any auxiliary transport mechanism would be relatively ineffective. It seems more likely, and would probably be an economical arrangement, that the uptake of the last traces of glucose might be linked to water movements. In this case any net water uptake would tend to concentrate the glucose in the gut lumen, which might then eventually diffuse passively into the haemolymph.

SUMMARY

1. The uptake of ^{14}C-labelled glucose, mannose and fructose from the alimentary canal of *Schistocerca gregaria* (Forsk.) has been studied using the dye Amaranth as a reference substance.

2. Absorption was confined to the mid-gut, the proportion absorbed by the caeca depending on the type of sugar and its concentration in the gut lumen.

3. The absorbed sugars were converted, in varying degrees, to trehalose which accumulated in the haemolymph. The extent of the conversion appeared to parallel the rate of absorption of the sugars at the various concentrations.

4. The sugars passed through the gut wall at similar rates in experiments in which the isolated alimentary canal was suspended in a large volume of circulating poisoned saline. The passage through the gut wall under these conditions was equivalent to the rapid absorption obtained *in vivo* when there was a rapid conversion to trehalose.

5. It is suggested that these observations support the hypothesis that the sugars are absorbed by diffusion across the gut wall and that the process is facilitated by the rapid conversion to trehalose in the haemolymph, which tends to maintain a steep concentration gradient.

6. At very low concentrations in the gut lumen, glucose was at a similar level to the relatively small amount of glucose in equilibrium with the trehalose in the haemolymph. When the specific activity of injected ^{14}C in the haemolymph approximated to that in the gut lumen the absorption of the labelled glucose from the mid-gut caeca was reduced at very low concentrations. Thus at these levels it is suggested that most of the absorption of labelled glucose can be attributed to an exchange with that in the haemolymph. At a concentration of 0·01 M/l. the proportion exchanging became negligibly small and the absorption of the labelled glucose molecules approximated to the net glucose absorption.

REFERENCES

BAAR, S. & BULL, J. P. (1953). Salt interference in sugar chromatography of urine. *Nature, Lond.*, **172**, 414–15.

DIMLER, R. J., SCHAEFER, W. C., WISE, C. S. & RIST, C. E. (1952). Quantitative paper chromatography of D-glucose and its oligosaccharides. *Analyt. Chem.* **24**, 1411–14.

EVANS, D. R. & DETHIER, V. G. (1957). The regulation of taste thresholds for sugar in the blowfly. *J. Inst. Physiol.* **1**, 3–17.

HIRST, E. L. & JONES, J. K. N. (1949). The application of partition chromatography to the separation of the sugars and their derivatives. *Disc. Faraday Soc.* **7**, 268–74.

HÖBER, R. (1899). Über Resorption im Dünndarm. *Pflüg. Arch. ges. Physiol.* **74**, 246–71.

HOWDEN, G. F. & KILBY, B. A. (1956). Trehalose and trehalase in the locust. *Chem. & Industr.* pp. 1453–4.

HOYLE, G. (1953). Potassium ions and insect nerve muscle. *J. Exp. Biol.* **30**, 121–35.

JERMYN, M. A. & ISHERWOOD, F. A. (1949). Improved separation of sugar on the paper partition chromatogram. *Biochem. J.* **44**, 402–7.

REID, A. F. (1947). Cited by Kamen, M. in *Radioactive Tracers in Biology*. New York: Academic Press.

TREHERNE, J. E. (1957). Glucose absorption in the cockroach. *J. Exp. Biol.* **34**, 478–85.

TREHERNE, J. E. (1958*a*). The absorption of glucose from the alimentary canal of the locust, *Schistocerca gregaria* (Forsk.). *J. Exp. Biol.* **35**, 297–306.

TREHERNE, J. E. (1958*b*). The mechanism of sugar absorption in the locust. *Proc. Fifteenth Int. Congr. Zool.* (in the Press).

TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, Lond.*, **166**, 444–5.

VERZÁR, F. (1931). Probleme und Ergebnisse auf dem Gebiete Darmresorption. *Ergebn. Physiol.* **32**, 391–471.

WYATT, G. R., & KALF, G. F. (1956). Trehalose in insects. *Fed. Proc.* **15**, 388.

YEAGER, J. F. & MUNSON, S. C. (1950). Blood volume of the roach *Periplaneta americana* determined by several methods. *Arthropoda*, **1**, 255–65.

STUDIES ON THE FEEDING AND NUTRITION OF *TUBEROLACHNUS SALIGNUS* (GMELIN) (HOMOPTERA, APHIDIDAE)

III. THE NITROGEN ECONOMY

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Since Tóth (1940) questioned that the dietary nitrogen supply of aphids is adequate for their growth and reproduction, considerable controversy has existed regarding the necessity and ability of aphids to supplement their dietary nitrogen supply with atmospheric nitrogen through the agency of their symbiotic micro-organisms. Few data are available, however, on the amounts of nitrogen actually ingested, excreted, and assimilated by aphids. The aim of the present investigation was to determine these data for *Tuberolachnus salignus* (Gmelin), and to resolve the controversy for this species.

MATERIAL AND METHODS

T. salignus was reared, handled, and caged as previously described (Mittler, 1957, 1958*a*). Potted, 2- to 4-year-old, *Salix acutifolia* Willd. trees, in various stages of leaf development, were used as host plants. The experiments were carried out in a greenhouse at a mean temperature of 20° C. under a photo-period of 16 hr.

Nitrogen assimilation of apterous and alate T. salignus. The following techniques were used to study the assimilation of nitrogen by a generation of apterous *T. salignus*: Twenty apterous adult aphids were confined in each of ten cages set up on the upright main stem (5–6 ft. long) of a single host plant. After 48 hr. the cages were opened, the adult aphids destroyed and 200 of their progeny of 800–1000 first-instar nymphs collected at random from all the cage sections. The average nitrogen content of the 200 first-instar nymphs was determined, after vacuum desiccation, by the standard micro-Kjeldhal steam distillation technique. The average nitrogen content of aphids in subsequent samples and that of cast skins were similarly determined.

The plant was then placed on its side on a bench and the pot wrapped in damp sacking to prevent the soil from spilling and drying out. In order to trap the skins cast by the nymphs which were allowed to remain and develop further on the stem a crystallizing dish containing water was placed under each of the ten cage sections. A daily count and collection of the cast skins established the percentage of all the nymphs on the stem which had moulted every 24 hr., and permitted the average nitrogen content of the skins cast by each instar to be determined. On the completion of each moult by all the nymphs 50–100 aphids were collected at random from the stem and their average nitrogen content determined. As the nitrogen assimilation of the apterous form of *T. salignus* was to be investigated exclusively in this experiment, all nymphs with wing pads (termed alate nymphs) were destroyed when the nymphs had completed their third ecdysis. The sample of fourth-instar nymphs therefore consisted entirely of apterous nymphs. The sample of third-instar nymphs may, however, have included a few alate nymphs as the aphids in this sample were not examined microscopically. External differences between potential apterous and alate individuals in the first and second instars are not apparent.

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When the apterous nymphs remaining on the stem had reached the adult stage, the plant was set upright again and ten of the young apterous adults were confined in each of ten stem cages. Daily thereafter the entire nymphal progeny of the adult aphids was counted and collected; the average nitrogen content of the nymphs born each day subsequently being determined. Periodic samples of ten adult aphids were collected at random from the ten cages in order to determine the average nitrogen content of the adult aphids throughout their life.

The daily amount of nitrogen expended per adult aphid through reproduction (termed the *daily nymphage*) was estimated by multiplying the number of nymphs produced per adult per day with the average nitrogen content of the nymphs born per day. The total amount of nitrogen expended in reproduction per adult aphid since the onset of reproduction (termed the *total nymphage*) was calculated each day by summing the daily nymphage values. The total amount of nitrogen assimilated per adult aphid was periodically established during the aphids' adult life by adding the appropriate total nymphage value to each available value of the aphids' average nitrogen content.

In order to compare the nitrogen assimilation of apterous and alate *T. salignus* the above techniques were also used. The period between which the first and last nymph in each instar moults was reduced, however, by allowing the twenty apterous adults initially confined in each of the ten cages to remain on the stem for 24 hr. only. Samples of first- and second-instar nymphs were consequently omitted so that a sufficiently large number of aphids should be available for sampling later during their development. Nymphs in third- and fourth-instar samples were examined under a binocular microscope in order to separate alate and apterous individuals. Nitrogen determinations were then made of the two forms in each instar. When all the nymphs had reached the fourth instar they were collected and the alate nymphs separated from the apterous. Twelve of the alate nymphs were then confined in each of five cages and seventeen of the apterous nymphs in each of five other cages, set up on the stem in alternate positions. It was readily possible thereafter to compare the further development of the two forms and to establish their daily nymphage and total nymphage under identical environmental conditions.

Nitrogen balance. It has previously been shown (Mittler, 1953, 1958*a*) that the total nitrogen (amino-acid and amide) concentration of the phloem sap of willows varies considerably with the developmental stage of their foliage. The hypothesis, that *the dietary nitrogen-supply received by* T. salignus *is adequate to support the* *aphid's nitrogenous requirements*, was therefore tested in five separate experiments in which willows having a mature foliage and a phloem sap poor in nitrogen were used in three of the experiments. A willow with a foliage in an active stage of growth and another with a foliage in an advanced stage of senescence were used in the other two experiments, both these willows having a phloem sap relatively rich in nitrogen.

Three cages, each 1 in. apart, were set up on the upright stem of each willow and twenty apterous adults confined in each cage. After 24 hr. the cages were opened, the adult aphids destroyed and their progeny collected from the uppermost and lowermost (later termed the two 'outer') of the three cage sections. The average nitrogen content of these 0–24 hr. old nymphs was subsequently determined. The willow was then placed on its side on a bench, the usual precautions being taken to maintain the soil moist within the pot. In order to collect all the honeydew droplets excreted by the nymphs remaining on the stem in the central cage section a 3 × 4 in. mica or glass plate was placed 1–2 in. under the aphids. Flies and other insects were denied access to the aphids or to the honeydew on the plate by enclosing the central cage section and the plate in a glass and plywood box, 6 in. cube, having a removable glass front panel. On each of the 7–21 days of an experiment the nymphs were counted and the plate carefully removed and a clean one put in its place. The honeydew on each plate was rapidly dried in a vacuum desiccator, in which the plates were also stored. The daily amount of sugar excreted per nymph was subsequently estimated by dissolving the honeydew on each plate in water and determining the total sugar concentration of the solution by the method of Morris (1948).

The method of Morris (1948) was also used to determine the total sugar concentrations of daily samples of freshly excreted honeydew. These samples, 5–10 $mm.^3$ in volume, were collected by means of a pipette from the surface of a waxed glass plate placed in the box for a few minutes each day. The methods of Tomkins & Kirk (1942) and Shaw & Beadle (1949) were used to determine the nitrogen concentrations of honeydew samples similarly collected and of phloem sap samples collected by the method previously described in detail (Mittler, 1958*a*). The amount of sugar excreted per nymph per day was divided by the corresponding concentration of sugar in the honeydew to give the volume of honeydew excreted per day; the total volume of honeydew excreted per nymph for a complete experiment was obtained by summing the daily values. The average nitrogen concentration of the phloem sap ingested by the nymphs during their development was determined by analysing periodic samples of the phloem sap ingested within 2–3 in. of the nymphs by some adult apterous aphids; the latter being allowed to feed for some hours per day on the stem in each of the two 'outer' cage sections.

At the end of the experiment the box was removed, the nymphs collected and their average nitrogen content determined. By subtracting the average nitrogen content of the 0–24 hr. old nymphs from this value, the amount of nitrogen assimilated per nymph was established; a small addition being made for the nitrogen in the skins cast by the nymphs. By multiplying the total volume of honeydew excreted per nymph by the difference in the average nitrogen concentrations of the phloem sap and honeydew the total amount of dietary nitrogen absorbed per nymph throughout the experimental period was established.

Nitrogen assimilation in relation to nitrogen level of phloem sap. In an experiment to determine simultaneously the nitrogen assimilation of *T. salignus* nymphs ingesting phloem sap of different nitrogen concentrations, two willows (*A* and *B*) with an actively growing foliage and a phloem sap 'rich' in nitrogen (greater than 0·1%, w/v), one willow (*C*) with a foliage in an advanced state of growth and a phloem sap 'medium' in nitrogen concentration (0·05–0·1%, w/v), and three willows (*D*, *E* and *F*) with a mature foliage and a phloem sap 'poor' in nitrogen (less than 0·05%, w/v) were used as host plants. Three cages were set up on each willow and twenty apterous adults confined in each cage. After 24 hr. the cages were opened and the adult aphids destroyed. When the nymphal progeny, which remained on the stems, were 7–8 days old their form, instar and average nitrogen content were determined.

It has previously been shown that the rate of excretion of *T. salignus* is a good index of the aphid's feeding rate (Mittler, 1957). In order, therefore, to compare the feeding rates of nymphs developing for 7–8 days on willow *B* with those of nymphs developing for the same period on willow *D* the rates of excretion of the nymphs on the two willows were estimated daily by determining the average number of honeydew droplets excreted by them per hour and the average volume of their honeydew droplets (Mittler, 1958*b*). The nitrogen concentrations of the honeydew excreted by the nymphs on the two willows were compared by matching the colour intensities produced by various dilutions of their honeydew spotted on filter-paper treated with ninhydrin. As in the previous experiment, the form, instar and average nitrogen content of the nymphs were determined when they were 7–8 days old.

RESULTS

Nitrogen assimilation of apterous and alate Tuberolachnus salignus

Fig. 1 shows the percentage of the nymphs in each instar which had moulted on each day of the experiment. The arrows *A–E* indicate when the four nymphal samples and the first apterous adult sample were taken for nitrogen determinations. The average nitrogen content of these samples is plotted in Fig. 2. The points *E–F* represent the average nitrogen content of the apterous aphids during their adult life. Points *X–Y* represent the total nymphage values. The total amount of nitrogen assimilated per apterous aphid throughout its nymphal and adult life is indicated by the points *A–E–G*. It may be noted that there is no abrupt change in the rate of nitrogen assimilation of apterous *T. salignus* at the onset of reproduction. After 9–10 days of reproduction the apterous aphids had given birth to thirty to thirty-five young per aphid and their total nymphage was equal to the average nitrogen content of their bodies.

Fig. 3 shows the percentage of apterous and alate nymphs in the third and fourth instars which had moulted on each day of the experiment. The arrows *C* and *D* indicate when nymphal samples of each form were taken, and the arrow *E* indicates

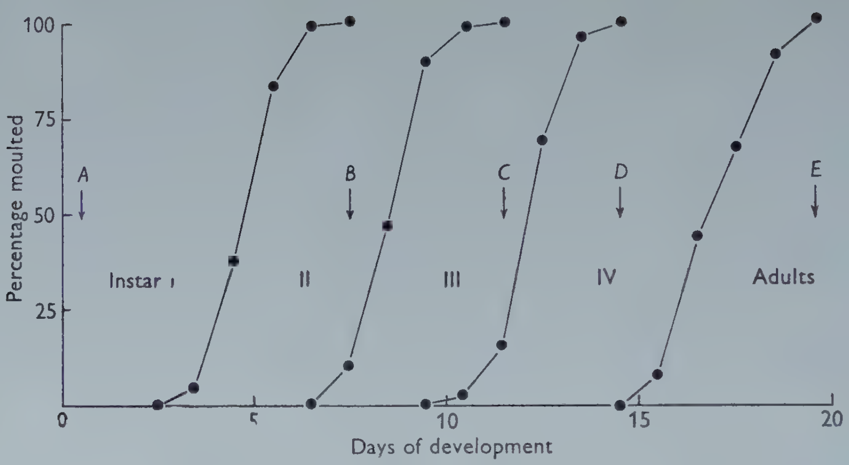

Fig. 1. Percentage of apterous nymphs moulting in first to fourth instars. Arrows indicate times of sampling.

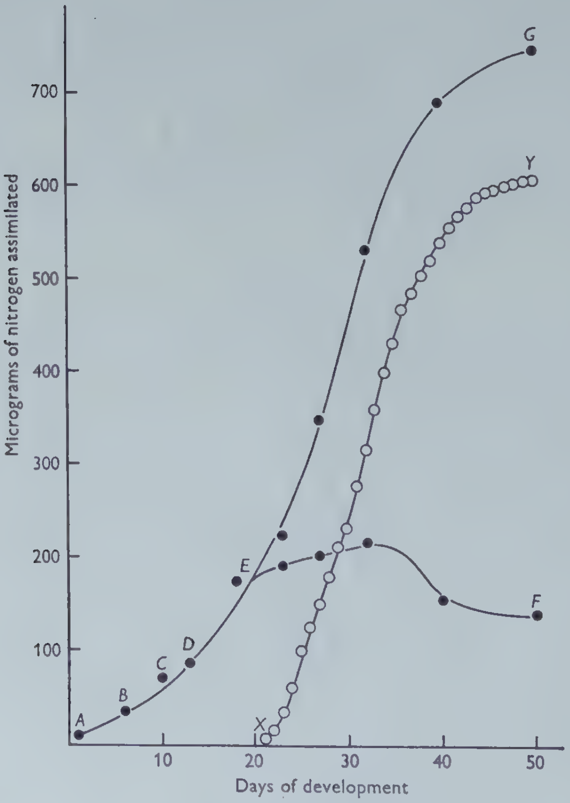

Fig. 2. Nitrogen assimilation of apterous *Tuberolachnus salignus*. For explanation see text.

when the first apterous adult sample was collected. In Fig. 4 the values of the average nitrogen content of the apterous nymphs and adults are represented by the points *C–E–F*, the total nymphage values of the apterous adults by the points *X–Y* and the total amount of nitrogen assimilated by the apterous nymphs and adults by the points *C–E–G*. The corresponding values for the alate nymphs and adults are represented by the points *C–P–Q*, *S–T* and *C–P–R*.

It is apparent from Fig. 3 that the alate third-instar nymphs moulted approximately 1 day after the apterous third-instar nymphs, and that the alate fourth-instar nymphs took 2–3 days longer to reach the adult stage than the apterous fourth-instar nymphs. As in the previous experiment, apterous adults began to bear young approximately 2–3 days after their final moult; alate adults, however, did not start to bear young until 6–7 days after their final moult. This period of adult development may be called the *pre-reproductive* period. Whereas the average nitrogen content of apterous and alate nymphs in the third and fourth instars differed only slightly, the nitrogen content of the apterous adults at the onset of their reproduction was almost twice as great as that of the alate adults when they began to bear young. The following observations may throw some light on these differences.

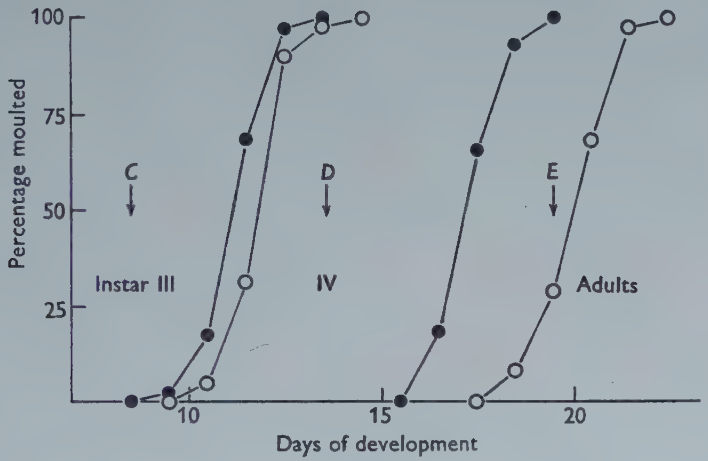

Fig. 3. Percentage of apterous (—●—) and alate (—○—) nymphs moulting in third and fourth instars. Arrows indicate times of sampling (see text).

The apterous adults tended to wander about in the cages for several hours on the first day of their adult life, but had settled to feed by the second day. This was indicated by honeydew droplets which began to accumulate on the cage walls at this time. The abdomen of these young apterous adults appeared swollen, and on dissecting some of them as many as seventy to eighty embryos in all stages of development were found in each aphid. Some dozen of the embryos in each aphid apparently were ready for birth.

The alate adults spent the first 3–4 days of their adult life sitting on the stem or cage walls. During this period few honeydew droplets were observed in the cages and only a few of the aphids were observed to have their stylets inserted into the stem, indicating that they were not ingesting any appreciable amount of food. The abdomen of each of the aphids was slender and contained only about thirty small embryos. On the fourth day of their adult life the aphids began to excrete as much honeydew as was being excreted by the apterous adults. After 2–3 days of feeding the abdomen of the alate adults had become distended and the aphids gave birth to their first young. Within 5 days of reproduction the aphids gave birth to an average of fifteen nymphs each and their total nymphage was equal to the average nitrogen content of their bodies. It is apparent from Fig. 4 that the total nymphage rate of the alate adults during the first 8 days of their reproduction does not differ greatly from the total nymphage rate which the apterous adults maintained during more than twice that period. The total number of young born per alate adult was forty-two compared to an average of eighty young to which the apterous adults gave birth. The reproductive life of the apterous adults was 22 days, however, compared to 15 days of reproduction by the alate adults.

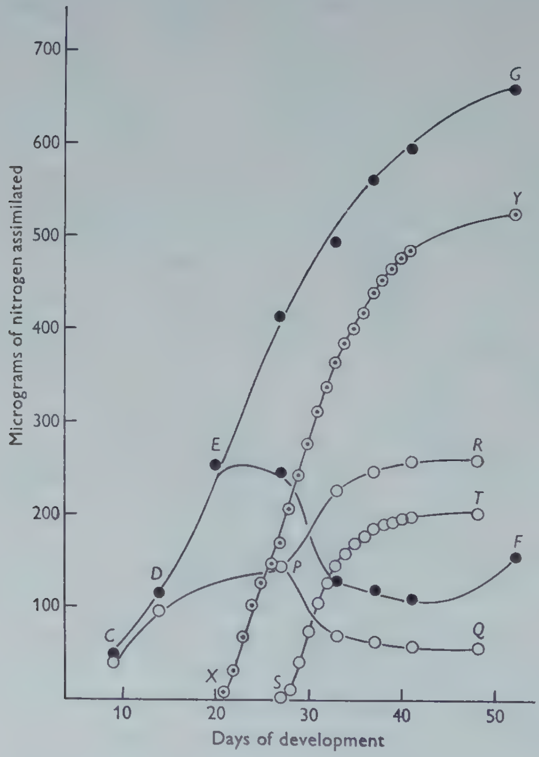

Fig. 4. Nitrogen assimilation of apterous and alate *Tuberolachnus salignus*. For explanation see text.

During the last week of their adult life the apterous adults gave birth to very few young and their average nitrogen content increased somewhat. It was noted that they continued to excrete large amounts of honeydew during this (*post-reproductive*) period and that their abdomens remained distended. However, not more than four embryos were found in each aphid dissected. Instead, their abdomen was found to be packed with thousands of deeply pigmented globules. The abdomen of alate adults appeared shrunken at the end of their reproductive period and was found to be filled with large wax-like concretions.

Nitrogen balance

Table 1 summarizes the results obtained in each of the five nitrogen balance experiments. It may be noted that the estimated amounts of nitrogen which the nymphs absorbed from the phloem sap they ingested during their development on each of the five willows do not differ greatly from the amounts of nitrogen actually assimilated by the aphids, the discrepencies falling within the 10–20% error to which the estimations were subject. From the volumes of honeydew excreted it appears that (per μg. of nitrogen assimilated by them) *T. salignus* nymphs ingest a relatively larger volume of sap poor in nitrogen than of sap rich in nitrogen. It is also clear that the nitrogen lost by excretion is not necessarily negligible compared to that ingested and cannot therefore be neglected when computing the nitrogen balance (cf. Smith, 1948).

Table 1. *Balance sheet of nitrogen ingested, excreted and assimilated by* Tuberolachnus salignus *nymphs*

Experiment ...	1	2	3	4	5
Foliage of willow ...	Senescent	Actively growing	Mature	Mature	Mature
Duration of experiment (days)	22	9	7	11	11
Amount of nitrogen assimilated per nymph (μg.)	87·50	173·2	46·96	73·89	58·82
Total volume of honeydew excreted per nymph (mm.^{3})	102·4	261·6	130·1	200	300
Nitrogen conc. of phloem sap (w/v)	0·128	0·122	0·052	0·039	0·032
Nitrogen conc. of honeydew (w/v)	0·050	0·055	0·016	0·0–0·01	0·0–0·01
Amount of nitrogen absorbed per nymph (μg.)	78·9	175·3	46·8	58–78	66–99
% nitrogen absorbed from phloem sap	61	55	69·3	74–100	69–100
Volume of honeydew excreted per μg. of nitrogen assimilated (mm.^{3})	1·17	1·52	2·78	2·7	5·1

Nitrogen assimilation in relation to nitrogen level of phloem sap

Table 2 sets out for comparison the average nitrogen content of nymphs which had developed for the first 7–8 days of their lives on each of six willows differing in the nitrogen concentration of their phloem sap. The number of nymphs on each willow in the second, third and fourth instars is stated, as well as the number and percentage of alate individuals amongst the third- and fourth-instar nymphs on each willow. It may be noted that the average nitrogen content of the 7- to 8-day-old nymphs on the 'nitrogen rich' willows *A* and *B* was almost ten times greater than that of the nymphs on the 'nitrogen poor' willows, *D*, *E* and *F*. The nymphs on plants *D*, *E* and *F* were, moreover, not much bigger than they were at birth and many of them were still in the second instar. The nymphs on plants *A* and *B*, on the other hand, were of 'normal' size, had all reached the third instar and several of them had already reached the fourth instar. It is also interesting to note that the percentage of alate nymphs is lowest on willows *A* and *B*.

Table 2. *Development and nitrogen assimilation of nymphs in relation to nitrogen level of phloem sap*

Phloem sap nitrogen level ...	Rich		Medium	Poor		
Plant	*A*	*B*	*C*	*D*	*E*	*F*
Average nitrogen content of 7- to 8-day-old nymphs (µg.)	109·0	116·0	46·8	14·5	11·7	11·5
No. of nymphs in each instar	40, 3rd; 9, 4th	141, 3rd; 32, 4th	166, 3rd	63, 3rd	35, 2nd; 14, 3rd	78, 2nd; 6, 3rd
No. of alate 3rd and 4th instar nymphs	5	34	83	26	17	8
% alate 3rd and 4th instar nymphs	10·2	19·7	50	41·3	54·8	57·1

Table 3. *Development and nitrogen assimilation of nymphs in relation to nitrogen level of phloem sap*

Phloem sap nitrogen level	Plant	Instar reached	Ratio of apterae to alatae	% alate	Total vol. of honeydew excreted (mm.^{3})	Nitrogen assimilated (µg.)	Vol. of honeydew excreted per µg. of nitrogen assimilated (mm.^{3})
Rich	*B*	3rd–4th	32:13	23·6	110	104·9	1·06
Poor	*D*	3rd	15:46	66·8	140	5·7	24·4

Table 3 sets out the results obtained in another experiment in which nymphs were again allowed to develop for 7–8 days after their birth on willows *B* and *D*. The amino-acid concentration of the honeydew excreted by the aphids feeding on willow *B* was found to be twenty-five times greater than that excreted by the aphids on willow *D*. The nymphs on willow *D* had all reached the third instar, although they were not much bigger than they were at birth and had assimilated almost no nitrogen during the 7–8 days of their development. The average nitrogen content of the nymphs on willow *B*, on the other hand, had increased twenty-fold since their birth and a few of them had already reached the fourth instar.

It may also be noted that the average volume of honeydew excreted per µg. of nitrogen assimilated by the nymphs on willow *D* was considerably larger than that excreted by the nymphs developing on willow *B*. Table 4 summarizes the honeydew excretion data for the nymphs in the first, second and third instars on each willow. It is immediately apparent that the excretory activity and hence also the feeding activity of the nymphs on willow *D* is considerably greater than that of the nymphs on willow *B*; the nymphs on willow *D* having a higher rate of excretion and a higher frequency of excretion, but a lower droplet volume (in consequence of their small size), than the nymphs on willow *B*.

Table 4. *Honeydew excretion of nymphs in relation to nitrogen level of phloem sap*

Instar	Phloem sap nitrogen level	Plant	Frequency of excretion (drops/hr.)	Droplet size (mm.^{3})	Rate of excretion (mm.^{3}/hr.)
1st	Rich	*B*	1·5	0·071	0·106
	Poor	*D*	11·3	0·055	0·622
2nd	Rich	*B*	2·68	0·136	0·364
	Poor	*D*	9·86	0·077	0·759
3rd	Rich	*B*	3·18	0·305	0·969
	Poor	*D*	6·2	0·173	1·073

DISCUSSION

In 1912 Peklo examined the mycetocytes of aphids and concluded that they contain symbiotic micro-organisms resembling *Azotobacter* bacteria, a group of bacteria known to be responsible for the fixation of atmospheric nitrogen in the root nodules of leguminous plants. This conclusion has recently been upheld by Peklo (1953) to counter claims made by Lanham (1952*a*, *b*) that the particles contained in the mycetocytes of aphids are not symbiotic bacteria but cell particulates. Assuming, however, that mycetocytes do contain symbiotic *Azobacter* bacteria Tóth (1940, 1946, 1952) claimed that these micro-organisms also provide aphids with reduced atmospheric nitrogen. The evidence for and against the fixation of atmospheric nitrogen by aphids may be discussed under following headings.

In vitro *fixation*. Tóth (1946, 1952) reported that the nitrogen content of nutrient media in which breis of various aphid species were incubated, increased by 100–150% in 24 hr., the only source of nitrogen being the air. Smith (1948) repeated these experiments in detail but could not detect any *in vitro* fixation of atmospheric nitrogen. Koch (1952) showed, however, that a pure culture of the symbiotic bacteria of a coccid (*Pseudococcus citri*) reduces atmospheric nitrogen and synthesizes six of the eight amino-acids which occur in the coccid's blood.

In vivo *fixation*. Smith (1948) exposed young feeding colonies of *Myzus persicae* and *Doralis* (*Aphis*) *fabae* to an atmosphere of oxygen and nitrogen containing 31·5 % of the stable isotope ^{15}N. No trace of the isotope was detected in the aphids after they had been exposed to such an atmosphere for 2–3 days. It might be supposed, however, that under the conditions of the experiment the aphids were receiving an abundant dietary nitrogen supply and hence not forced to draw upon atmospheric nitrogen, or that they are only forced to do so during reproduction.

As *in vitro* nitrogen fixation does not occur unless the nitrogen content of the culture medium is very low, Fraenkel (in a discussion of Tóth, 1952) questioned the ability of symbionts to fix atmospheric nitrogen in the nitrogen rich milieu of an insect cell.

Respiratory quotient. Tóth & Wolsky (1941) demonstrated that living *Pterocallis juglandis* have a R.Q. of 0·86. This value was claimed to indicate that protein is practically the sole substrate being oxidized by the aphids; the implication being that reduced atmospheric nitrogen is available to the aphids in large enough quantities for them to oxidize it. It may be noted, however, that a R.Q. of 0·86 may also indicate an oxidation by fasting aphids of a nitrogen-free substrate composed of carbohydrates and fats in the proportion 7:3.

Nitrogen content of sap and honeydew. Data on honeydew and phloem sap from divers sources led Tóth (1940, 1946) to suppose that the nitrogen concentration of the honeydew excreted by aphids is greater than that of the phloem sap they ingest; this supposition implying that reduced atmospheric nitrogen is available to aphids in such quantities that they have to excrete some of it. Michel (1942) indicated that the honeydew excreted by *Lachnus roboris* contains twice as much nitrogen per dry weight as the phloem sap of the aphid's host plant. These results may be questioned, however, on the grounds that the phloem sap was collected directly from oak stems, whereas the honeydew was collected from the aphids colonizing severed oak branches. Lindemann (1948) has since clearly demonstrated that the honeydew excreted by *Cryptomyzus ribis* has a consistently lower nitrogen content than the phloem sap of *Ribes rubrum* branches colonized by the aphids. Such a relationship between phloem sap and honeydew has also been amply confirmed for *T. salignus* with respect to each amino-acid and amide as well as with respect to the total nitrogen contained by these fluids (Mittler, 1953, 1958*a*). If some of the amino-acids in honeydew were the products of atmospheric nitrogen fixation one might expect a few of them at least to differ from the amino-acids found in the phloem sap. This was not found to be the case for *T. salignus* honeydew and *Salix* phloem sap. It is interesting to note, however, that Gray (1952) found six to seven amino-acids in the honeydew of *Pseudococcus brevipes* which were not present in sap expressed from the coccid's host plant. The possibility that these amino-acids are general metabolic products of the coccid cannot be ruled out.

Nitrogen assimilation. Tóth (1940) claimed that the nitrogen expended per day in terms of young by an adult *Aphis sambuci* at the peak of its reproduction is equal to the aphid's own weight of nitrogen, and that the dietary nitrogen supply is inadequate for this. The results of the present study show that apterous *T. salignus* expend only about one-tenth of their own weight of nitrogen per day when maintaining a steady rate of reproduction. Although alate *T. salignus* expend one-fifth of their own weight of nitrogen per day during their shorter period of reproduction this is not due to a higher total nymphage rate, but due to the lower nitrogen content of the alate adults. The rate at which nitrogen is assimilated during reproduction by the alate adults is in fact less than that of the apterous adults. During reproduction both forms draw to some extent on nitrogen assimilated by them during their nymphal life; the alate adults apparently doing so right at the onset of reproduction. This is readily understood when one considers that embryos are already being formed during the nymphal development of apterous and alate *T. salignus*, and that autolysis of the wing muscles of the alate adults normally sets in shortly before they begin to bear their first young (Johnson, 1957). A limited amount of 'stored' nitrogen is therefore available and used up during reproduction.

Tóth (1940) showed that mycetocytes increase in number and that symbionts get dissolved in the body cavity of an aphid when it reproduces. This was interpreted to indicate that the aphid calls on its symbionts to supply it with atmospheric nitrogen for reproduction. Although there is a gradual increase in the rate at which nitrogen is assimilated by apterous *T. salignus* during their development it was noted that at the onset of reproduction there was no marked change in the assimilation rate. This indicates that apterous *T. salignus* when reproducing do not call upon a process of nitrogen assimilation differing from that responsible for their nymphal nitrogen assimilation. The marked change in the assimilation rate of alate *T. salignus* at the onset of their reproduction has already been discussed. It was felt, therefore, that any conclusions which might be drawn from the results of the experiments on the assimilation of dietary nitrogen by *T. salignus* nymphs would also apply to *T. salignus* adults when reproducing.

Lindemann (1948) pointed out that if aphids were able to draw on atmospheric nitrogen their growth should be independent of the nitrogen level of the phloem sap of their host plant. Lindemann found, however, that the size of *Cryptomyzus ribis* was positively related to the nitrogen concentration of the phloem sap of the host plant. The results of the present investigation also indicate that the size and nitrogen content of *T. salignus* depend directly on the nitrogen concentration and volume of the phloem sap ingested. *T. salignus* assimilate only as much nitrogen as they are able to absorb from the phloem sap ingested by them. Although certain aphid species may have evolved the ability to assimilate atmospheric nitrogen, no evidence for this has been found from a study of *T. salignus*, which is a species belonging to the 'primitive' aphid family—the Lachnidae.

The results further indicate that a higher proportion of aphids develop wings on a diet poor in nitrogen than on one relatively rich in nitrogen. This is in agreement with the findings of Evans (1938). It may be speculated that apterous adult aphids, which may be considered to be neotenous individuals, are produced in response to a nitrogen-rich food supply from nymphs whose reproductive development is in advance of the differentiation of their adult characters.

SUMMARY

1. The investigation concerns the ingestion, excretion, and assimilation of dietary nitrogen by *Tuberolachnus salignus* (Gmelin) developing on *Salix* trees in various stages of leaf development.

2. Apterous and alate forms of *T. salignus* differ in their rates of development and nitrogen assimilation and in their behaviour.

3. The aphids assimilate considerably more nitrogen when developing on willows having a phloem sap rich in nitrogen than on willows having a phloem sap relatively poor in nitrogen.

4. For a given amount of nitrogen assimilated the aphids ingest a larger volume of a phloem sap poor in nitrogen than of a phloem sap rich in nitrogen.

5. Nitrogen balance experiments have shown that *T. salignus* nymphs assimilate only as much nitrogen as they are able to absorb from their food.

6. The evidence for and against the fixation of atmospheric nitrogen by aphids through the agency of their symbionts is briefly discussed. *T. salignus* do not supplement their dietary nitrogen supply by fixing atmospheric nitrogen.

REFERENCES

EVANS, A. C. (1938). Physiological relationships between insects and their host plants. I. The effect of the chemical composition of the plant on reproduction and production of winged forms in *Brevicoryne brassicae* L. (Aphididae). *Ann. App. Biol.* **25**, 558–72.

GRAY, R. A. (1952). Composition of honeydew excreted by pineapple mealybugs. *Science*, **115**, 129–33.

JOHNSON, B. (1957). Studies on the degeneration of the flight muscles of alate aphids. I. A comparative study of the occurrence of muscle breakdown in relation to reproduction in several species. *J. Ins. Physiol.* **1**, 248–56.

KOCH, A. (1952). Über die Physiologie intrazellulärer Symbionten. *Zbl. Bakt.* **158**, 363–6.

LANHAM, U. N. (1952*a*). Observations on the supposed intracellular symbiotic microorganisms of aphids. *Science*, **115**, 459–60.

LANHAM, U. N. (1952*b*). Mitochondria or microorganisms? *Science*, **116**, 332–3.

LINDEMANN, C. (1948). Beitrag zur Ernährungsphysiologie der Blattläuse. *Z. vergl. Physiol.* **31**, 112–33.

MICHEL, E. (1942). Beiträge zur Kenntnis von *Lachnus* (*Pterochlorus*) *roboris* L., einer wichtigen Honigtauerzeugerin an der Eiche. *Z. angew. Ent.* **29**, 243–81.

MITTLER, T. E. (1953). Amino-acids in phloem sap and their excretion by aphids. *Nature, Lond.*, **172**, 207.

MITTLER, T. E. (1957). Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). I. The uptake of phloem sap. *J. Exp. Biol.* **34**, 334–41.

MITTLER, T. E. (1958*a*). Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew. *J. Exp. Biol.* **35**, 74–84.

MITTLER, T. E. (1958*b*). The excretion of honeydew by *Tuberolachnus salignus* (Gmelin) (Homoptera: Aphididae). *Proc. R. Ent. Soc. Lond.* (A), **33**, 49–55.

MORRIS, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science*, **107**, 254–5.

PEKLO, J. (1912). Über symbiotische Bakterien der Aphiden. *Ber. dtsch. bot. Ges.* **30**, 416–9.

PEKLO, J. (1953). Microorganisms or mitochondria? *Science*, **118**, 202–6.

SHAW, J. & BEADLE, L. C. (1949). A simplified ultra micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples less than 1·0 cu.mm. *J. Exp. Biol.* **26**, 15–23.

SMITH, J. D. (1948). Symbiotic micro-organisms of aphids and fixation of atmospheric nitrogen. *Nature, Lond.*, **162**, 930–31.

TOMPKINS, E. R. & KIRK, P. L. (1942). Quantitative drop analysis. XVI. An improved diffusion method for total nitrogen. *J. Biol. Chem.* **142**, 477–85.

TÓTH, L. (1940). The protein metabolism of the aphids. *Ann. Mus. Hist-nat. Hungar. Pars Zool.* **33**, 167–71.

TÓTH, L. & WOLSKY, A. (1941). Gaswechsel und respiratorischer Quotient bei den Aphiden. *Zool. Anz.* **136**, 99–103.

TÓTH, L. (1946). *The Biological Fixation of Atmospheric Nitrogen.* Budapest: Hungarian Museum of Natural Sciences.

TÓTH, L. (1952). The role of nitrogen-active microorganisms in the nitrogen metabolism of insects. *Tijdschr. Ent.* **95**, 43–62.

A SURVEY OF THE ENZYMES FROM THE GASTRO-INTESTINAL TRACT OF *HELIX POMATIA*

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INTRODUCTION

The digestive tract of *Helix pomatia* is known to contain many digestive enzymes. Holden & Tracey (1950) list a wide variety of substrates which are attacked by *Helix* sp. digestive juices, but their data are mainly qualitative and are drawn from publications by a number of authors.

Extracts from the gastro-intestinal tract of *H. pomatia* are particularly rich in cellulase and have frequently been used in cytological work to digest plant cell walls because of this cytase activity. The present investigation was carried out both to assess the relative amounts of cellulase and other enzymes in such an extract before attempting to purify cellulase from it, and to see how the efficacy of snail cytase is correlated with the range of carbohydrases present.

MATERIALS AND METHODS

(*a*) *Preparation of an enzymically active extract from* Helix pomatia

Specimens of *H. pomatia* in a resting condition, with a thick calcareous epiphragm closing the shell, were dissected by the method of Keilin (1956). The digestive tracts were excised and cut into 1–2 cm. lengths. They were homogenized with the gastro-intestinal tract contents and an equal volume of ice-cold distilled water in a Potter homogenizer (Potter & Elvehjem, 1936) which was fitted with a Perspex pestle and surrounded by ice. The homogenate was centrifuged at 19,000 *g* at 4° C. and the clear brown supernatant decanted. It was dialysed for 16 hr. in cellophane dialysis tubing against a large volume of ice-cold distilled water and any precipitate which formed was removed by centrifuging. The resulting solution was used in the enzyme assays.

200 snails yielded about 100 ml. of this solution, which contained approx. 10 mg./ml. protein N, and was stored for up to 6 months at $-15°$ C. The solution showed three boundaries when examined in a Tiselius electrophoresis apparatus (Perkin Elmer Model 38). At pH 6·8 and I 0·2, the mobilities of these boundaries in the descending limb of the apparatus were $0{\cdot}372 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$, $0{\cdot}854 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ and $2{\cdot}45 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$.

(*b*) *Preparation of substrates*

Tween 20 was freed from free fatty acids by the method of Archibald (1946).

β-Methyl cellobioside was prepared by a four-stage process. Cellobiose octa-acetate was prepared from 20 g. cotton-wool (Dorée, 1947) and was converted to hepta-acetyl β-methyl cellobioside by the method of Pacsu (1930). This was deacetylated (Zemplén, 1926) and the product recrystallized. 3 g. of crystalline β-methyl cellobioside were obtained, m.p. 191° C. (uncorr.); $[\alpha]_D$ $-18{\cdot}4°$. Zemplén & Gerecs (1930) give m.p. 193° C. and $[\alpha]_D$ $-19{\cdot}1°$ for this compound.

Chitosan acetate was prepared by the method of Meyer & Wehrli (1937).

Cellofas B, a sodium carboxymethyl cellulose derivative, was supplied by I.C.I. Ltd. The degree of substitution of a sample labelled 'low' viscosity grade was found to be 0·46 by a colorimetric method (Eyler, Klug & Diephuis, 1947). An approximately 1 % solution was prepared. The powder was added to distilled water, stirred mechanically for about 4 hr. and the mixture centrifuged at 800 *g* for 30 min. to remove traces of insoluble material. The amount of Cellofas B in the supernatant was found by a dry-weight determination on a small sample and the concentration adjusted by the addition of distilled water to give a 0·5 % solution which could be stored up to 2 months at 2° C.

Bleached cotton linters were kindly given by Lansil Ltd., Shipley, Yorks. They were swollen with phosphoric acid at 0° C. for 10 min. (Walseth, 1952). The degree of polymerization of the treated linters was estimated by the method of Atchison (1943) and was found to be 500.

Laminarin was kindly given by Dr D. J. Manners.

Wheat straw xylan and *walnut xylan* were prepared by the method of Dorée (1947).

Snail galactan was prepared by the method of May (1934).

Yeast mannan was prepared by the method of Haworth, Heath & Peat (1941), and *ivory nut mannan A* and *ivory nut mannan B* by the method of Aspinall, Hirst, Percival & Williamson (1953).

Yeast glucan was prepared by the method of Bell & Northcote (1950). The other substrates used were supplied commercially.

(*c*) *Methods of measuring enzymic activities*

Proteinases. These were assayed by the method of Anson (1938).

Carbohydrases. Invertase and *maltase* were estimated by the manometric method of Keilin & Hartree (1948) using a glucose oxidase preparation supplied by the Sigma Chemical Company and labelled 'Crude, on inert base'. *Lactase* was estimated, using the same impure notatin preparation, by the method described by Hestrin, Feingold & Schramm (1955).

Cellobiase was estimated by a reducing sugar method, since a glucose oxidase preparation free from cellobiase could not be obtained. 5 ml. of 0·5 % β-methyl cellobioside solution were incubated in a stoppered boiling tube at 37° C. with 0·5 ml. M buffer at an appropriate pH and 4·0 ml. distilled water. 0·5 ml. of the enzyme solution was added and the mixture was incubated with shaking for 20 min. 2 ml. of the incubation mixture were then added to 2 ml. of alkaline copper-sulphate solution (Somogyi, 1952) and heated on a boiling-water bath for 15 min. The solution was cooled rapidly to room temperature, 2 ml. arseno-molybdate reagent (Nelson, 1944) added, the volume of the solution made up to 20 ml. with distilled water and the colour intensity at 620 mμ read on a Unicam colorimeter (Model S.B. 350) against a blank of boiled enzyme incubated in the same way. The glucose in the sample was then calculated from a calibration curve, using 50–200 μg. samples of glucose.

α-Amylase and β-amylase were estimated by the method of Noelting & Bernfeld (1948).

Xylanase, *yeast mannanase*, *laminarinase* and *galactanase* were estimated in the same way as cellobiase activity, the amount of reducing sugar being calculated in each case using a calibration curve prepared from the appropriate monosaccharide. *Ivory nut mannan A* was used in 0·1% solution, since it was insoluble at higher concentrations.

Pectinase was estimated viscometrically. 5 ml. of 5% apple pectin solution were mixed in an Ostwald viscometer with 0·5 ml. M buffer of the required pH and 4·0 ml. distilled water and placed in a constant-temperature bath at 30° C. When the contents of the viscometer had reached the temperature of the bath, the time for the liquid to flow through the capillary of the viscometer was noted. 0·5 ml. of the enzyme solution was added and the solution mixed by blowing air through it. The time taken for the solution to flow through the capillary of the viscometer was measured at intervals from the time of mixing.

Chitosanase was measured viscometrically by a similar method to that used for pectinase.

Cellulase was assayed by measuring the reducing sugar formed from Cellofas B solutions, as in the determinations of cellobiase, and also by the production of reducing sugars from phosphoric acid-swollen cotton linters (an insoluble substrate). For these assays, 0·2000 g. samples of oven-dried, phosphoric acid-swollen cotton linters were shaken at 25° C. in 20 ml. screw-capped bottles with 1 ml. M buffer at the required pH value and distilled water to give a total volume of 19 ml. In some cases 2 ml. 0·1% crystalline bovine serum albumin (Whitaker, 1952) were added before the water. 1 ml. of the enzyme solution was added and the incubation continued for 17 hr.; 2 ml. of the mixture were then added to 2 ml. alkaline copper reagent (Somogyi, 1952) and the assay completed in the same way as that of cellobiase activity.

Yeast glucanase and activity towards *ivory nut mannan B* were estimated by similar procedures to that used for cellulase on insoluble substrates. Calibration curves were prepared from the appropriate monosaccharide and were used to calculate the degradation.

Chitinase was measured in a similar way to cellulase on the insoluble substrate, but the incubation period was extended to 40 hr. and the acetyl glucosamine formed was estimated by the method of Aminoff, Morgan & Watkins (1952).

Lipases were measured on Tween 20 by the method of Bier (1955).

(*d*) *Reagents*

All chemicals used were of A.R. quality.

Phosphate buffers were used between pH 8·0 and 5·75, acetate between pH 5·75 and 3·5 and glycine/HCl between pH 3·5 and 2·0.

(*e*) *Nitrogen determinations*

Micro-Kjeldahl digestion was carried out as described by Chibnall, Rees & Williams (1943) followed by distillation and titration.

EXPERIMENTAL RESULTS

(*a*) *Proteinases*

Table 1 shows the proteinase activity of the enzymically active extract from the digestive tract of *Helix pomatia* in the presence and absence of potassium cyanide solution. An enzyme solution which liberated 1 mM. of tyrosine when it was incubated with nine times its volume of 2·2% denatured haemoglobin was given an activity of 1 (Anson, 1938).

Table 1. *Proteinase activity of* Helix pomatia *digestive tract extract*

pH value of incubation in 0·05 M buffer	2 M-KCN added/10 ml. incubation mixture (ml.)	Enzymic activity/ml. digestive tract extract
7·5	—	41·6
7·5	0·1	41·6
5·6	—	3·0
5·6	0·1	3·0
2·5	—	16·2
2·5	0·1	16·2

(*b*) *Carbohydrases*

Table 2 gives the results of the quantitative determinations of the activity of the carbohydrases in the extract from *H. pomatia* digestive tract.

β-Methyl cellobioside was used to assay cellobiase activity and Cellofas B to assay cellulase activity. It was necessary to confirm that the enzymic degradations of these substrates proceeded in the same way as those of cellobiose and cellulose, by cleavage of 1:4-β-glycosidic bonds between adjacent glucose units.

9·5 ml. of the assay medium were incubated for 16 hr. at 37° C. with 0·5 ml. undiluted snail digestive tract extract. The solution was then evaporated at 60° C. under reduced pressure, to give 0·1 ml. of a thin oil.

The concentrated solution from the hydrolysis of β-methyl cellobioside was investigated chromatographically and electrophoretically. 5μl. were examined chromatographically on Whatman No. 1 filter-paper which had been sprayed with saturated sodium borate solution and allowed to dry. The chromatogram was developed for 16 hr. at 25° C., using propanol azeotrope, which had been saturated with sodium borate, as the solvent system. The paper was dried and spots coloured by the silver nitrate reagent of Trevelyan, Proctor & Harrison (1950). It showed spots at 14 and 19·5 cm. from the origin. A methyl glucose marker ran 19 cm. and β-methyl cellobioside and glucose markers ran 14 cm. from the origin. In the electrophoretic examination, 5 μl. were used and the electrophoresis carried out in 0·5 M sodium borate solution (Fuller & Northcote, 1956). After 1 hr. at 33 V./cm. and 20 mA., the spots were coloured by silver nitrate. Two spots were seen from the hydrolysate, at 0·5 and 5·0 cm. from the origin towards the anode. The latter corresponded to the glucose marker and the former to β-methyl glucose and β-methyl cellobioside markers, these last two running together in this experiment. No evidence that cellobiose was present was found from either the electrophoretic or the chromatographic experiments.

Table 2. *Carbohydrase activities of* Helix pomatia *digestive tract extract*

Substrate	Main monosaccharide constituents	Links in substrate	pH value for optimum activity	Temperature of assay (° C.)	mg. reducing sugar formed/hr./ml. extract
Sucrose	Glucose, fructose	1:2-αβ	5·6	37	1·1
Lactose	Galactose, glucose	1:4-β	7·25	37	0·9
Maltose	Glucose	1:4-α	5·0	37	1·1
β-Methyl cellobioside	Glucose	1:4-β	4·75	37	3
Wheat straw xylan	Xylose	1:4-β	4·75	37	40
Walnut xylan	Xylose	1:4-β	4·5	37	40
Snail galactan	Galactose	—	4·0–7·5	37	0
Yeast mannan	Mannose	1:2-α 1:3-α	5·0	37	30
Ivory nut mannan A	Mannose	1:4-β	4·5	37	80
Ivory nut mannan B	Mannose	1:4-β	4·5	25	7*
Yeast glucan	Glucose	1:2-β 1:3-β	5·5	25	2*
Laminarin	Glucose	1:3-β	5·1	37	160
Soluble starch	Glucose	1:4-α	6·8	25	0·78
		1:6-α	4·8	25	2·5
Snail glycogen	Glucose	1:4-α	6·8	25	1·9
		1:6-α	4·8	25	11·1
Chitin	Acetyl glucosamine	1:4-β	4·75	25	12
Cellofas B	Glucose, Carboxymethyl glucose	1:4-β	5·5	37	1090
Phosphoric acid-swollen cotton linters	Glucose	1:4-β	5·5	25	11*

* Crystalline bovine serum albumin added to give a final concentration of 2 mg./20 ml. assay medium.

The concentrated solution from the enzymic hydrolysis of Cellofas B was examined electrophoretically. The buffer used was pyridine/ethyl acetate/water at pH 6·5 (Michl, 1951). The field strength was approx. 50 V./cm., the current of about 35 mA. being passed for 1 hr. After electrophoresis the paper was dried and sprayed with aniline phthalate (Consden & Stanier, 1952) to detect reducing sugars or with a 1% solution of bromphenol blue to detect acidic spots. Reducing sugar spots were seen at distances 1 cm. from the origin towards the cathode and at 7, 11 and 17 cm. towards the anode; a glucose marker moved 1 cm. towards the cathode. The spots which migrated towards the anode also stained with bromphenol blue, and were attributed to carboxymethyl sugar derivatives. This interpretation was confirmed by examining a partial acid hydrolysate of Cellofas B by electrophoresis, when the spots which stained with both aniline phthalate and bromphenol blue were seen to move the same distances towards the anode as in the enzymic hydrolysate.

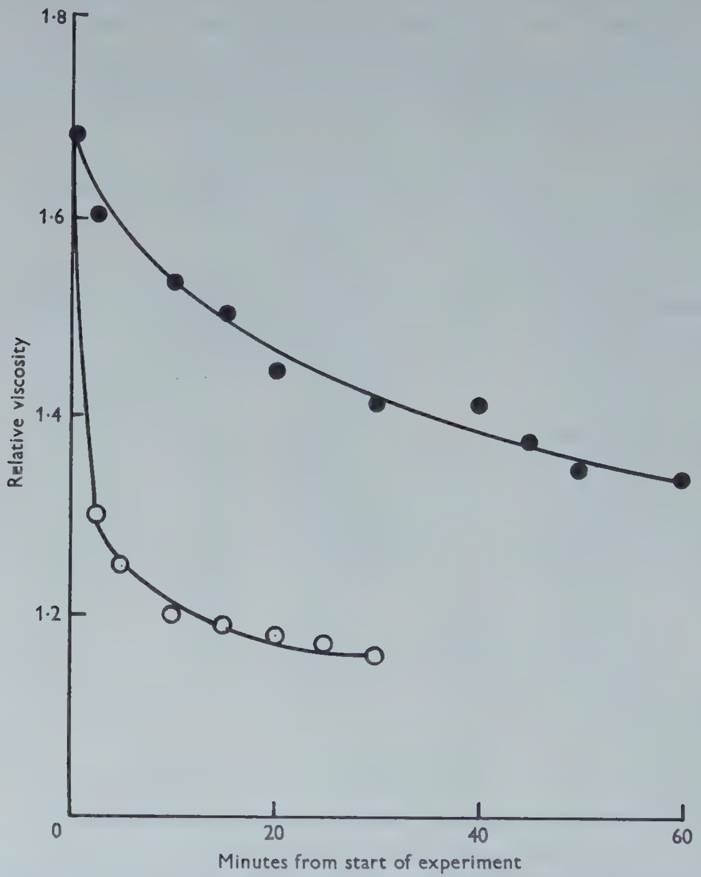

Fig. 1. Enzymic degradation of apple pectin. Full circles—*Helix pomatia* digestive tract extract. Open circles—Pectinol 100 D. Experiments were carried out at 30° C. In the final solutions there were 5 mg./ml. apple pectin.

Fig. 1 shows the decrease in viscosity of an apple-pectin solution when incubated with snail digestive tract extract and compares the rate of fall with that found when a fungal pectinase solution (Pectinol 100 D, Rohm & Haas) of similar protein content was incubated with a similar solution. For both pectinase solutions, optimum activity was found at pH 4·0.

Fig. 2 illustrates the fall of viscosity of a chitosan acetate solution when it was incubated with undiluted *H. pomatia* digestive tract solution at the pH for optimum activity, pH 5·6.

(*c*) *Lipases*

There were 1050 units of the lipase activity in each ml. of undiluted digestive tract extract. The definition of the unit of lipase activity was that used by Bier (1955).

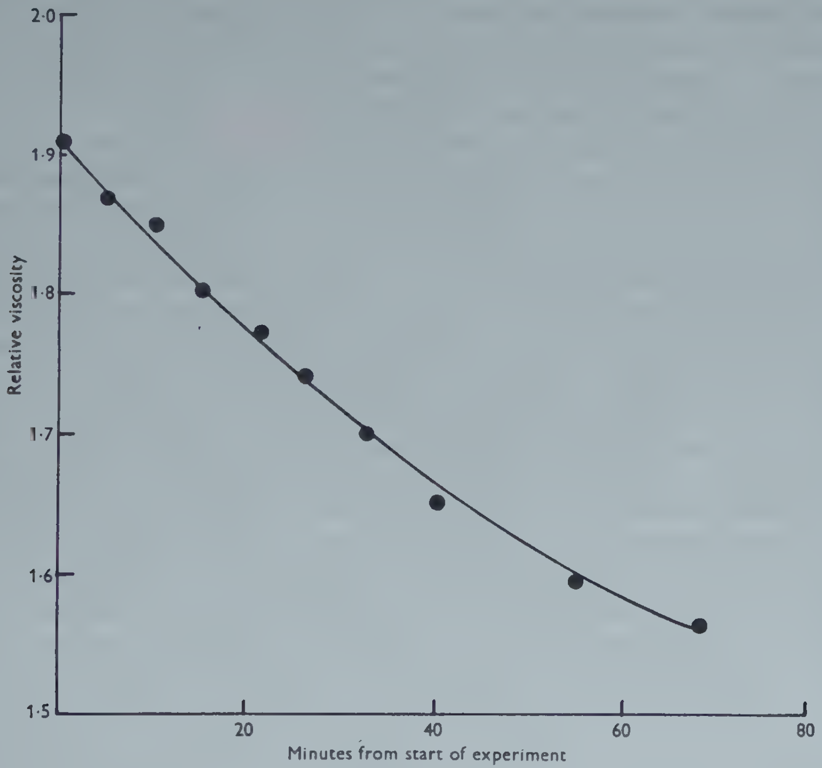

Fig. 2. Enzymic degradation of chitosan acetate solution by *Helix pomatia* digestive tract extract. The experiment was carried out at 30° C. There were 5 mg./ml. chitosan acetate in the final solution.

DISCUSSION

The experiments which have been reported show that a variety of substrates are attacked by a preparation from the digestive tract of *H. pomatia*. All these substances are present in the snail's diet under natural conditions or are closely similar to such compounds. The enzyme preparation showed three boundaries in the Tiselius electrophoresis apparatus, so that there were not less than three soluble proteins in the solution.

The proteinases present were mainly of the intercellular, digestive type; their activities were not stimulated by the addition of cyanide to the assay system, although the prosthetic groups of cathepsins often contain sulphydryl groups and are activated by –SH reagents (Smith, 1951). The relative lack of proteinases in the extracts was perhaps not surprising, since the snail is herbivorous. The stability of the carbohydrases when the solution was kept for long periods must derive, at least in part, from the small amounts of proteinases present, for even native carbohydrases would be attacked to some extent by proteinases.

Since a carbohydrase is usually specific with reference both to the linkage and to the monosaccharide constituents of the polymer it splits, it is probable that most of the carbohydrates chosen were degraded by discrete enzyme systems. However, the possibility that a number of substrates were attacked by the same enzyme cannot be ignored. A number of the polysaccharide substrates were broken down much more rapidly than any of the disaccharides tested.

The linkages in yeast mannan are mainly α (Haworth, Hirst & Isherwood, 1937) and those in ivory nut mannans are β (Klages, 1934*a*, *b*) which could account for the observed differences in pH optima for the two substrates and for the different extents of degradation. The use of ivory nut mannan A and ivory nut mannan B enables a direct comparison to be made of the amount of degradation of soluble and insoluble molecules. Both these mannans are made up from two types of molecules, one consisting of chains of 1:4-β linked mannopyranose units and the other of identical chains terminated by a galactopyranose residue, the only difference between the two mannans being the lengths of the chains (Aspinall *et al.* 1953). From Table 2 it can be seen that reducing sugar is released from ivory nut mannan B at 8% of the rate at which it is released from ivory nut mannan A. A larger difference was found when cellulase was assayed on Cellofas B and phosphoric acid-swollen cotton linters, possibly because the latter substance was enzymically degraded without the production of reducing sugars and because the two substrates are of different chemical compositions. It is interesting that bleached cotton linters were not degraded by *H. pomatia* digestive tract extract with the release of reducing sugars until they had been swollen with phosphoric acid.

The only substrate which could not be degraded under the experimental conditions used was snail galactan. This observation is in contrast to the result of Weinland (1953) who found a weak galactanase activity which was optimum at pH 4·0.

From the results given, it is clear that cellulose and some hemicellulose components of plant cell walls will be attacked by snail cytase preparations. Care is necessary, therefore, in the interpretation of structural studies using such preparations; the study of lignified material is further complicated by the report (Holden & Tracey, 1950) that ligninase is present in *H. pomatia* digestive tract.

The significant lipase activity of the digestive tract extract is probably an important factor in its utility as a cytase, contributing to the breakdown of lipoprotein structures in cell walls.

SUMMARY

1. The digestive tract of the snail has been isolated and a soluble extract prepared from it and its contents. A quantitative analysis of the proteinases, lipases and twenty different carbohydrase activities present in this extract has been made.

2. Snail galactan was not degraded by the enzymic preparation.

3. The use of the preparation for cytological studies has been discussed.

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REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). Studies in immunochemistry. II. The action of dilute alkali on the N-acetyl hexosamines and the specific blood-group mucoids. *Biochem. J.* **51**, 379–89.

Anson, M. L. (1938). Estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *J. Gen. Physiol.* **22**, 79–89.

Archibald, R. M. (1946). Determination of lipase activity. *J. Biol. Chem.* **165**, 443–8.

Aspinall, G. O., Hirst, E. L., Percival, E. G. V. & Williamson, I. R. (1953). The mannans of ivory nut (*Phytelephas macrocarpa*). Part I. The methylation of mannan A and mannan B. *J. Chem. Soc.* pp. 3184–8.

Atchison, J. (1943). The influence of cooking and bleaching upon the chain length distribution of the carbohydrate fraction in pulp wood. *Paper Trad. J.* **116**, 23–34.

Bell, D. J. & Northcote, D. H. (1950). The structure of a cell-wall polysaccharide of baker's yeast. *J. Chem. Soc.* pp. 1944–7.

Bier, M. (1955). Lipases. Ed. Colowick, S. P. and Kaplan, N. O., *Methods in Enzymology*, **1**, 627–42. New York.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354–9.

Consden, R. & Stanier, W. M. (1952). Ionophoresis of sugars on paper and some applications to the analysis of protein polysaccharide complexes. *Nature, Lond.*, **169**, 783–5.

Dorée, C. (1947). *The Methods of Cellulose Chemistry*, 2nd ed. London.

Eyler, R. W., Klug, E. D. & Diephuis, F. (1947). Determination of degree of substitution of sodium carboxymethylcellulose. *Analyt. Chem.* **19**, 24–7.

Fuller, K. W. & Northcote, D. H. (1956). A micro method for the separation and determination of polysaccharides by zone electrophoresis. *Biochem. J.* **64**, 657–63.

Haworth, W. N., Heath, R. L. & Peat, S. (1941). The constitution of yeast mannan. *J. Chem. Soc.* pp. 833–42.

Haworth, W. N., Hirst, E. L. & Isherwood, F. A. (1937). Polysaccharides. Part XXIV. Yeast mannan. *J. Chem. Soc.* pp. 784–91.

Hestrin, S., Feingold, D. S. & Schramm, M. (1955). Hexoside hydrolases. Ed. Colowick, S. P. and Kaplan, N. O., *Methods in Enzymology*, **1**, 231–58. New York.

Holden, M. & Tracey, M. V. (1950). A study of enzymes that can break down tobacco-leaf components. 2. Digestive juice of *Helix* on defined substrates. *Biochem. J.* **47**, 407–14.

Keilin, D. & Hartree, E. F. (1948). The use of glucose oxidase (notatin) for the determination of glucose in biological materials and for the study of glucose-producing systems by manometric methods. *Biochem. J.* **42**, 230–8.

Keilin, J. (1956). Helicorubin and cytochrome *h*. Purification and physical properties. *Biochem. J.* **64**, 663–76.

Klages, F. (1934*a*). Zur Kenntnis der Steinnuss-mannane. 1. Die Konstitution von Mannan A. *Liebigs Ann.* **509**, 159–82.

Klages, F. (1934*b*). Die Konstitution von Mannan B. *Liebigs Ann.* **512**, 185–94.

May, F. (1934). Chemische und biologische Untersuchungen über Galaktogen. *Z. Biol.* **95**, 277–97.

MEYER, K. H. & WEHRLI, H. (1937). Comparaison chimique de la chitine et de la cellulose. *Helv. chim. acta*, **20**, 353–62.

MICHL, H. (1951). Über Papierionophoresese bei Spannungsgefallen von 50 Volt/cm. *Mh. Chem.* **82**, 489–93.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**, 375–80.

NOELTING, G. & BERNFELD, P. (1948). Sur les enzymes amylolytiques. III. La β-amylase: dosage d'activite et controle de l'absence d'α-amylase. *Helv. chim. acta*, **31**, 286–90.

PACSU, E. (1930). Action of tetrachloride on derivatives of sugars. IV. Transformation of hepta-acetyl-beta-methylcellobioside to the alpha form and the preparation of alpha-methyl cellobioside. *J. Amer. Chem. Soc.* **52**, 2571–5.

POTTER, V. R. & ELVEHJEM, C. A. (1936). A modified method for the study of tissue oxidations. *J. Biol. Chem.* **114**, 495–504.

SMITH, E. L. (1951). Proteolytic enzymes. Ed. Sumner, J. B. & Myrbäck, K., *The Enzymes*, **1**, 793–872. New York.

SOMOGYI, M. (1952). Notes on sugar determination. *J. Biol. Chem.* **195**, 19–23.

TREVELYAN, W. R., PROCTOR, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, Lond.*, **166**, 444–5.

WALSETH, C. S. (1952). The influence of the fine structure of cellulose on the action of cellulases. *Tech. Pap. Pulp Pap. Ind., N.Y.*, **35**, 233–8.

WHITAKER, D. R. (1952). An effect of proteins and proteoses on the cellulase of *Myrothecium verrucaria*. *Science*, **116**, 90–4.

WEINLAND, H. (1953). Über den Galaktogenabbau durch Fermente in vitro Versuche an Wienbergschnecken (*Helix pomatia*). *Biochem. Z.* **324**, 19–31.

ZEMPLÉN, G. (1926). Abbau der reduzierenden Biosen. I. Direkte Konstitutions-Ermittlung der Cellobiose. *Ber. dtsch. chem. Ges.* **59**, 1254–8.

ZEMPLÉN, G. & GERECS, A. (1930). Einwirkung von Quecksilbersalzen auf Aceto-halogenzucker. IV. Mitteil. Direkte Darstellung der Alkylbioside der α-Reihe. *Ber. dtsch. chem. Ges.* **63**, 2720–9.

THE MECHANISM OF MARINE OSMOREGULATION IN THE LAMPERN (*LAMPETRA FLUVIATILIS* L.) AND THE CAUSES OF ITS BREAKDOWN DURING THE SPAWNING MIGRATION

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INTRODUCTION

An earlier contribution (Morris, 1956) described the osmoregulatory responses of lamperns to sea water during the time that the animals are engaged on a spawning migration into fresh water. Only fresh-run animals were found to be able to osmoregulate in dilute sea-water solutions hypertonic to their blood, and even in these creatures the osmotic responses were very variable. These facts suggested that the mechanism responsible for maintaining the blood hypotonic to sea water was breaking down as the need arose for the development of a new osmoregulatory mechanism to allow the animal to exist in fresh water.

The present study describes the osmoregulatory processes of fresh-run animals which were found to be capable of maintaining ion and water balance in dilute sea water and also attempts to analyse the reasons for the breakdown of the mechanism which takes place in other fresh-run animals. There were indications from previous work that the mechanism is similar to that already described for marine teleosts by Homer Smith (1930). Because of this, the experiments to be described were mainly designed to test whether the lampern was able to swallow sea water and excrete chloride extra-renally.

The gill epithelia of some fresh-run lamperns contain cells which are similar to those described from the gills of marine teleosts as chloride excretory cells, and in the lampern these cells are replaced by others as the animal matures (Morris, 1957). An attempt has been made to assess the number of chloride excretory cells which were present in individual experimental animals and this assessment has then been related to the osmotic responses of the animal.

MATERIAL AND METHODS

The experiments were conducted on a single haul of lamperns taken from the River Trent as early as possible in their spawning migration to ensure that a large proportion of animals capable of osmoregulating in sea water would be present. The animals were transported in damp grass from Newark-upon-Trent to Nottingham as soon as possible and those showing the slightest sign of damage were discarded.

The experimental procedure outlined below was successfully completed on eighteen animals. These were taken in groups of five or six individuals, since this was the most convenient number to handle at one time. All experiments were completed within a month from the time the animals were captured.

Animals were first adapted to 33% sea water for a minimum period of 40 hr. They were weighed before and after this period to obtain a preliminary assessment of their osmoregulating capabilities from the extent to which they were able to maintain water balance in a solution which is only slightly hypertonic to their blood. They were then anaesthetized in chlorbutol prior to their swallowing-capacities being tested by the method devised by Smith (1930). According to this method the animal is immersed in a solution of a dye which cannot be absorbed by the gut, the gills or the skin. The gut is blocked to prevent loss of swallowed water, and after a suitable period of time the amount of water swallowed and absorbed can be estimated by comparing the concentration of dye in the gut residue with that in the surrounding sea water. The contents of the gut and kidneys were expelled from the lamperns by gentle abdominal massage, and the hind end of the gut was then blocked by inserting a solid cone of soft Polythene through the anus into the gut. At the same time the urinary papilla was ligatured for urine collection during the period of the experiment. This method was chosen both for its simplicity and because the urine output is known to be low when animals are immersed in hypertonic solution (Morris, 1956). The method is only valid for low urine outputs because the volume of the urinary tract of the lampern is small (1–2 ml.).

The animals were allowed to recover in 33% sea water before they were transferred to a solution of 50% sea water containing phenol red (25 mg./l.). After about 24 hr. they were again anaesthetized and weighed, and the gut and its content were removed before the volume of the residual fluid within the gut was measured. Blood samples were then taken and, after the serum had been separated by centrifugation, the serum, the gut content, urine and sea water were analysed for chloride and freezing-point depression by the methods described previously (Morris, 1956).

Δ_t indicates the measured freezing-point depression. Δ_{Cl} indicates the freezing-point depression due to monovalent chloride, as given by the relation 293 mM./l. $\mathrm{NaCl} \equiv 1^\circ\,\mathrm{C}.\Delta$ (Krogh, 1939).

In order to determine the amount of sea water swallowed, an aliquot of the gut content was diluted to a known volume with water containing a little 0·1 N sodium hydroxide to develop the colour of the phenol red. The colour intensity of this solution was compared in a Duboscq colorimeter with that of a known solution in sea water diluted to almost the same colour intensity by a known volume of alkaline solution. In this way it is possible to determine the relative concentrations of phenol red and knowing the volume of residual fluid within the gut to calculate the amount of sea water swallowed and absorbed by the animal. As Smith points out, this method of measuring swallowed water would be open to question if phenol red were absorbed by the animal by way of the gut, gills or skin. Examination of the centrifuged plasma failed to reveal signs of phenol red even after the addition of alkali, nor could any be found in the tissues. The efficiency of the gut block was also checked in a few cases by placing animals which were known to have swallowed sea water into 1 l. of fresh 50% sea water for some time. No phenol red could be detected in the water.

In order to assess the number of chloride excretory cells whole gill pouches were taken from each individual at the end of the experiments and were fixed in Helly's fluid. They were then post-chromed for 3 days at 37° C. to preserve phospholipid material (Baker, 1945) and were later embedded in paraffin wax. Sections four to five μ thick were cut from the outer surface of the gill pouch in a plane parallel to the long axis of the gill filaments. The first ten sections were discarded, and the two immediately following were used; one was stained by the Kull method for mitochondria (Baker, 1945), the other was coloured by the Sudan Black method for phospholipid as described by Thomas (1948). The chloride excretory cells are clearly distinguished by either method (Morris, 1957). The sections were examined using a one-sixth objective and no attempt was made to count the number of chloride excretory cells because of the difficulty of preparing strictly comparable sections owing to differences of curvature between individual gill pouches. Instead, representative sections from each animal were ranked in different categories according to the number of cells. Four groups were recognized: those containing 'many', 'few', 'very few' and 'nil'. There was no difficulty in distinguishing sections belonging to the extreme groups, only those in the groups 'few' and 'very few' proved difficult to place. Because of this, sections from both of these groups, stained by the same method, were ranked in order of the relative abundance of chloride excretory cells. The rankings were carried out quite independently on the two sets of slides and gave a 'coefficient of correspondence' (Moroney, 1951) of 0·72 indicating a good agreement between separate rankings. Separation into the two groups was then carried out on the ranked slides.

The experiments relating to gut diameter and swallowing capacity were conducted on maturing animals during the same season. Swallowing capacity in 50% sea water was determined by the method described above; gut diameter was measured in the pancreatic region of the gut (Barrington, 1944), since it is here, at the junction of the oesophageal and the intestinal regions, that the gut seems to become occluded as the animal matures. The animals were dissected to reveal the oesophagus, after which this was injected with a solution of warm, coloured gelatine. The solution passed backward into the pancreatic region of the gut and eventually flowed out through the cut end of the intestine. The pancreatic region was then dissected out, fixed and dehydrated in alcohol, after which it was cleared and mounted in cedar wood oil. The maximum and minimum diameter of the coloured gelatine plug was then measured by means of a microscope equipped with a micrometer eyepiece.

RESULTS

The results of eighteen successful experiments are recorded in Table 1. Experiments have been ranked according to the level of the plasma freezing-point depression (Δ_i) of the animals and are numbered in this order. Examples of typical responses have been selected and these are summarized in histogram form in Fig. 1.

Table 1. *The osmotic responses of lamperns immersed in 50% sea water*

Exp. no.	Initial weight (g.)	% change of wt. in 50% sea water	Environment and body fluids	Δ_t (° C.)	Calcd. Δ_{Cl} (° C.)	% $\frac{\Delta_{Cl}}{\Delta_t}$	Collection period (hr.)	Amount of fluid (ml.)	Fluid content of gut (ml.)			Chloride excretory cell assessment
									Intake	Absorbed	Residue	
1	70·8	+4·0	Sea water	0·925	0·820	88·8						
			Gut fluid	0·795	0·415	52·0	19·5	2·1	8·4	6·3	2·1	Many
			Plasma	0·565	0·390	69·0						
			Urine	—	—	—	6·5	Nil				
2	74·0	+3·0	Sea water	0·975	0·815	83·5						
			Gut fluid	0·580	0·425	73·0	19·5	3·0	13·2	10·2	3·0	Many
			Plasma	0·570	0·415	73·0						
			Urine	—	—	—	6·5	Nil				
3	79·6	+2·5	Sea water	1·00	0·885	88·5						
			Gut fluid	0·580	0·400	69·0	9·0	1·5	6·0	4·5	1·5	Many
			Plasma	0·575	0·400	69·5						
			Urine	—	—	—	9·0	Nil				
4	75·1	+2·5	Sea water	0·990	0·850	85·8						
			Gut fluid	0·585	0·465	79·5	23·0	1·8	7·6	5·8	1·8	Many
			Plasma	0·580	0·400	68·9						
			Urine	—	—	—	6·5	0·025				
5	64·1	−10·5	Sea water	0·965	0·820	84·9						
			Gut fluid	—	—	—	20·5	0·08	0·18	0·10	0·08	Many
			Plasma	0·660	0·450	68·1						
6	72·8	−10·0	Sea water	0·980	0·820	83·6						
			Gut fluid	—	0·615	—	27·0	0·38	1·9	1·52	0·38	Few
			Plasma	0·680	0·510	75·0						
7	80·65	−6·3	Sea water	0·925	0·820	88·6						
			Gut fluid	—	—	—	18·5	0·10	0·33	0·23	0·10	Few
			Plasma	0·740	0·510	68·4						
8	73·0	−10·9	Sea water	0·985	0·835	84·7						
			Gut fluid	0·700	0·520	74·2	24·0	1·38	5·08	3·70	1·38	Few
			Plasma	0·740	0·530	71·6						
9	50·0	−8·4	Sea water	0·925	0·820	88·6						
			Gut fluid	—	—	—	19·0	Nil	Nil	Nil	Nil	Very few
			Plasma	0·755	0·545	72·0						

Table 1 (*cont.*)

Exp. no.	Initial weight (g.)	% change of wt. in 50 % sea water	Environment and body fluids	Δ_t (° C.)	Calcd. Δ_{Cl} (° C.)	% $\frac{\Delta_{Cl}}{\Delta_t}$	Collection period (hr.)	Amount of fluid (ml.)	Fluid content of gut (ml.)			Chloride excretory cell assessment
									Intake	Absorbed	Residue	
10	100·2	−7·9	Sea water	0·980	0·835	84·5	27·0	2·08	9·5	7·42	2·08	Few
			Gut fluid	0·730	0·565	77·3						
			Plasma	0·770	0·585	75·9						
11	90·65	−4·1	Sea water	0·975	0·815	83·5	19·5	0·3	3·7	3·4	0·3	Few
			Gut fluid	—	0·440	—						
			Plasma	0·785	0·590	75·1						
12	76·7	−9·3	Sea water	0·970	0·825	85·0	23·0	0·40	0·4	—	0·4	Nil
			Gut fluid	—	0·650	—						
			Plasma	0·800	0·610	76·2						
13	70·5	—	Sea water	1·00	0·885	88·5	9·0	1·2	1·6	0·4	1·2	Few
			Gut fluid	0·830	0·705	85·0						
			Plasma	0·810	0·585	72·2						
14	68·9	−12·9	Sea water	0·970	0·825	85·0	23·0	0·9	0·9	—	0·9	Nil
			Gut fluid	0·790	0·635	80·0						
			Plasma	0·825	0·590	71·5						
15	88·5	−12·45	Sea water	0·990	0·850	85·8	19·0	1·6	5·8	4·2	1·6	Few
			Gut fluid	0·780	0·615	78·8						
			Plasma	0·840	0·590	70·2						
			Urine	0·680	0·445	65·5	4·5	0·72				
16	81·7	−8·0	Sea water	0·985	0·835	84·5	23·0	0·8	2·5	1·7	0·8	Nil
			Gut fluid	0·820	0·650	79·2						
			Plasma	0·840	0·610	72·6						
17	78·0	−10·0	Sea water	0·970	0·825	85·0	23·0	0·4	2·2	1·8	0·4	Nil
			Gut fluid	—	0·635	—						
			Plasma	0·845	0·635	75·0						
18	82·4	−11·7	Sea water	0·980	0·820	83·6	23·0	0·90	4·4	3·3	0·9	Nil
			Gut fluid	0·615	0·440	71·5						
			Plasma	0·870	0·655	75·0						

The experimental results obtained for each animal are represented by a maximum number of five blocks. The first four blocks refer to various fluids, as follows: (i) (Dashes) sea water swallowed. (ii) (Broken cross-hatching) fluid absorbed from gut. (iii) (Dots) fluid remaining in gut. (iv) (Unbroken cross-hatching) blood. The value of 5·7 ml. of blood per 100 g. found by Welcker and quoted by Reichert & Brown (1909) has been used to calculate the blood volume of each animal from its initial weight.

In the above blocks, the areas give a direct measure of the amounts of substance involved since Δ_t is given by total height; Δ_{Cl} is given by the height of the shaded area; ml./24 hr. is given by the width.

Block (v) is in fact a line rather than a block, whose height represents the chloride excretory cell assessment.

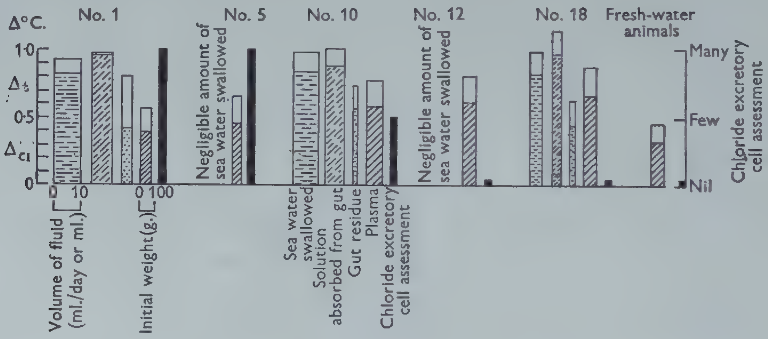

Fig. 1. The osmotic responses of selected fresh-run lamperns after immersion in 50 % sea water (for further explanation see text).

THE CRITERIA OF SUCCESSFUL OSMOREGULATION

Two factors have been taken into consideration when deciding whether an animal has been osmoregulating successfully. These are (1) the ability to keep the body weight relatively constant, and (2) the ability to maintain the plasma osmotic pressure well below that of the environment. Only four animals satisfied these conditions in the present experiments. The mean values for freezing-point depression, blood chloride and percentage change of weight from the initial weight for these animals are recorded in Table 2. Similar measurements made on other fresh-run animals during an earlier series of experiments (Morris, 1956, fig. 7) are also included for comparison. These animals were immersed in 33 % sea water (Δ_t=0·660° C.) for a minimum period of 48 hr.

As might be expected, the values for plasma freezing-point depression and chloride are somewhat lower in lamperns immersed in 33 % sea water than those immersed in 50 % sea water; the mean value of Δ = 0·572° C. for the plasma of these latter animals compares favourably with the value recorded by Burian (1910) for the blood of related *Petromyzon marinus* (Δ = 0·58° C.) living in sea water. This suggests that the lampern may also maintain its blood close to this concentration when living in sea water. The increase of plasma osmotic pressure (24 %) brought about by

Table 2. *The plasma freezing-point depression (Δ_t), plasma chloride (Δ_{Cl}) and percentage change of weight from initial weight of fresh-run lamperns osmoregulating in various environments*

Environment	No. of observations	Plasma Δ_t (° C.)	Calculated plasma Δ_{Cl} (° C.)	% $\frac{\Delta_{Cl}}{\Delta_t}$	% change from initial weight
Fresh water	10	0·456 ± 0·006	0·325 ± 0·006	71·3	—
33 % sea water (Δ_t=0·660° C.)	4	0·547 ± 0·011	0·359 ± 0·020	65·6	−2·5 ± 0·2
50 % sea water (Mean Δ_t=0·970° C.)	4	0·572 ± 0·003	0·404 ± 0·005	70·6	+3·0 ± 0·3

Table 3. *Water balance in lamperns osmoregulating in 50 % sea water*

Exp. no.	Initial weight of animal, g.	Water balance (change in weight minus gut residue)		Water swallowed		Residual fluid in gut		Calculated water absorbed		% of swallowed water absorbed	Urinary output		Calculated extra-renal loss	
		g.	g./100 g./day	ml.	ml./100 g./day	ml.	ml./100 g./day	ml.	ml./100 g./day		ml.	ml./100 g./day	ml.	ml./100 g./day
1	70·8	+0·75	+1·3	8·4	14·7	2·1	3·7	6·3	11·0	75	Nil	—	5·55	9·7
2	74·0	−0·75	−1·3	13·2	22·1	3·0	5·1	10·2	17·0	77	Nil	—	10·95	18·3
3	79·6	+0·5	+1·8	6·0	20·1	1·5	5·0	4·5	15·1	75	Nil	—	4·0	13·3
4	75·1	+0·1	+0·14	7·6	10·5	1·8	2·4	5·8	8·1	77	0·025	0·13	5·6	7·83

transferring animals from fresh water to 50% sea water is of the same order as that recorded by Keys (1933) for the common eel (19%), when this animal is transferred from fresh water to full-strength sea water.

THE MECHANISM OF MARINE OSMOREGULATION

Water balance

Table 3 summarizes the data relating to water balance in the four osmoregulating animals. All of these results have been calculated in the following way. During Exp. 4, the lampern (initial weight, 75 g.) gained 1·9 g. over a period of 23 hr. immersion in 50% sea water. At the end of the experiment the gut contained 1·8 ml. of residual fluid to which most of the weight increase must be attributable. The condition of water balance at the end of the experiment must therefore have been +0·1 g. Measurements of the volume of residual fluid within the gut (1·8 ml.) and the relative concentration of phenol red showed that the animal had swallowed 7·6 ml. of sea water of which 5·8 ml. must have been absorbed. Since 0·1 g. of this contributed to the increase of weight and 0·1 ml. was excreted as urine, it follows that 5·6 ml. of water must have been lost by an extra-renal route, presumably by osmotic withdrawal through the permeable surface of the gills and, to a lesser extent, through the skin.

The rate of extra-renal loss of water is very much higher than that measured for the eel. The figures obtained by Smith (1930) give a loss of 6·25 ml. of water per 100 g. per day from an eel living in 100% sea water, an environment of twice the osmotic concentration of that in which the lamperns were kept. These findings confirm earlier comparisons of the relative permeabilities of the external surfaces of the two animals (Morris, 1956). In earlier experiments, as in the present ones, fresh-run lamperns showed a great deal of variability with regard to external surface permeability and, moreover, mature animals showed an increase in permeability when compared with fresh-run lamperns. It was, therefore, argued that permeability had already started to increase in fresh-run animals and that this process continued as the animals approached maturity. This possibly explains why the lampern is unable to osmoregulate in high concentrations of sea water even during the earliest stages of its spawning migration. The relatively high extra-renal loss may also account for the abnormally low urine outputs recorded for all osmoregulating animals. These values are lower than those given for marine teleosts which appear to vary between 0·2 and 0·5 ml./100 g. per day (Smith, 1930; Grafflint 1931; Pitts, 1933) and they may be taken as an indication of water shortage brought about by the abnormally high extra-renal loss.

Ion balance

Only chloride concentration and freezing-point depression were measured during the present investigation so that only anions can be considered. The data relating to anion balance are summarized in Tables 4 and 5 and they have been calculated in the following manner. Let us consider first the behaviour of chloride during Exp. 1.

Table 4. *Chloride balance in lamperns osmoregulating in 50% sea water*

Exp. no.	Cl swallowed		Cl in gut residue		Calculated Cl absorbed		% of swallowed Cl absorbed	Calculated amount and average concentration of Cl solution absorbed		Urinary Cl	Calculated extra-renal loss of Cl	
	mM.	mM./100 g./day	mM.	mM./100 g./day	mM.	mM./100 g./day		mM.	ml./100 g./day		mM.	mM./100 g./day
1	2·02	3·53	0·26	0·45	1·76	3·08	87	6·3 of 279	11·0 of 279	Nil	1·76	3·08
2	3·15	5·28	0·37	4·64	2·78	0·44	87	10·2 of 271	17·0 of 271	Nil	2·78	4·64
3	1·56	5·22	0·18	0·59	1·38	4·63	89	4·5 of 307	15·1 of 307	Nil	1·38	4·63
4	1·89	2·60	0·25	0·33	1·64	2·27	87	5·8 of 283	8·1 of 283	—	—	—

Table 5. *Balance of anions other than chloride ('A') in lamperns osmoregulating in 50% sea water*

Exp. no.	'A' swallowed		'A' in gut residue		Calculated 'A' absorbed		% of 'A'	Calculated amount and average concentration of 'A' solution absorbed		Urinary 'A'
	mM.	mM./100 g./day	mM.	mM./100 g./day	mM.	mM./100 g./day		mM.	ml./100 g./day	
1	0·26	0·46	0·23	0·41	0·03	0·05	11	6·3 of 4·7	11·0 of 4·7	Nil
2	0·62	1·04	0·14	0·23	0·48	0·81	77	10·2 of 47·0	17·0 of 47·0	Nil
3	0·20	0·66	0·08	0·26	0·12	0·40	61	4·5 of 26·6	15·1 of 26·6	Nil
4	0·32	0·43	0·06	0·08	0·26	0·35	81	5·8 of 43·8	8·1 of 43·8	Nil

The animal swallowed 8·4 ml. of sea water during immersion in 50% sea water containing 240·0 mM. Cl./l., so that 2·02 mM Cl were taken into the gut. At the end of the experiment the gut residue amounted to 2·1 ml. and, since this contained 121·5 mM. Cl/l., the total amount of chloride in the gut residue becomes 0·26 mM. It follows from this that the animal absorbed 1·76 mM. Cl and, since this was absorbed at the same time as 6·3 ml. of fluid, the average strength of the chloride solution taken up becomes 279 mM./l. (Fig. 1). Because of the comparatively low level of blood chloride at the end of the experiment (144·5 mM./l.) it may be assumed that most of the chloride absorbed from the gut had been actively excreted by the animal into sea water containing roughly twice as much chloride as the blood and, since this particular animal produced no measurable quantity of urine, the excretion must have taken place by an extra-renal route.

Similar considerations apply to other osmoregulating animals, although in those cases where urine was produced it has been impossible to partition the excretion into renal and extra-renal routes because urinary chloride was not measured.

There is further evidence that chloride excretion takes place extra-renally. The osmoregulating mechanism was only partially effective in one of the animals studied (Table 1, Exp. 15) and here the lampern produced sufficient urine for it to be shown that the urine remained hypotonic to the blood. ($\Delta_{\text{plasma}} = 0{\cdot}840^\circ$ C., $\Delta_{\text{urine}} = 0{\cdot}680^\circ$ C.). The urinary chloride (130 mM./l.) was also maintained at a lower level than that of the blood (173·5 mM./l.) although the lampern was in obvious osmotic difficulties. This may be taken as an indication that the lampern kidney is similar to the kidneys of other lower vertebrates (Smith, 1932) and is unable to produce a hypertonic urine and conserve water in this way. The kidney is presumably orientated toward a fresh-water existence and thus continues to take up ions in spite of changed osmotic circumstances.

It is also possible to calculate the balance of non-chloride anions from the data available (Table 5 and Fig. 1, non-shaded areas). In sea water and the residual fluid of the gut these anions are presumed to consist mainly of sulphate, together with a little bicarbonate. The following method of calculation has been employed, using Exp. 1 again as an example. The freezing-point depression of 50% sea water in this case is equivalent to a total ionic concentration of 271 mM. NaCl/l. and, since the animal swallowed 8·4 ml. of sea water, this volume contained the equivalent of 2·28 mM. NaCl in total. 2·02 mM. of the anions are known to be chloride, so that the quantity of non-chloride anion swallowed was equivalent to 0·26 mM. NaCl. The same method of calculation can be applied to the gut residue (2·1 ml.: $\Delta = 0{\cdot}795^\circ$ C.) and here the non-chloride anion amounts to 0·23 mM. NaCl. The animal must therefore have absorbed 0·03 mM. of non-chloride anion, which represents about 11% of the quantity originally swallowed by the animal. Since the non-chloride anion was absorbed with 6·3 ml. of fluid, the average concentration of fluid absorbed is equivalent to 4·7 mM. NaCl.

The general pattern of the various factors involved during absorption from the gut can be seen by reference to Fig. 1 and Table 1. Considering chloride first, it is noticeable that in osmoregulating animals the concentration of chloride in the gut residue never drops below that of the blood. This suggests that the chloride ion enters the body fluids by a process of physical diffusion and, because the average concentration of chloride absorbed is somewhat higher than that of sea water which is swallowed, the chloride must enter faster than the water which accompanies it. Absorption of non-chloride fraction of the swallowed sea water could also be explained in the same way, since the inorganic anions (mainly sulphate) contributing to this fraction of the gut residue are certain to be in greater concentration than in the blood, which contains a relatively high proportion of organic materials acting as anions (Robertson, 1954). The rate of entry of non-chloride anion, however, varies between different individuals (Tables 1, 4). In Exp. 1 it enters at a much slower rate than either chloride or water and hence accumulates in the gut. The conditions in Exps. 3 and 2 are similar, although the rate of entry is much faster in these animals. In the last of the osmoregulating animals (Exp. 4) the concentration of non-chloride anion in the gut residue ($\Delta_A = 0{\cdot}120^\circ$ C.) is less than that of the swallowed sea water and must therefore enter faster than the water absorbed at the same time. If, as seems likely, the processes of anionic absorption in the lampern gut are brought about by diffusion, then it may be assumed that these substances are either exchanged for other anions, or that cations accompany them. Judging from Smith's work (1930) on marine teleosts the latter process seems much more likely, since in these creatures monovalent ions (Na^+ and Cl^-) are absorbed preferentially, thus leaving the divalent ions (Ca^{2+}, Mg^{2+} and SO_4^{2-}) behind. The data presented for Exp. 1 suggest that a similar process takes place in the lampern and, since other animals show an increasing tendency to take up divalent anions, it may be that gut permeability becomes altered in some way during the migration.

The fate of the divalent ions taken up by these animals is uncertain. It is unlikely that they remain in the blood stream because the proportion of chloride to total ions remains almost the same in animals osmoregulating in 50% sea water as in fresh-run animals taken from fresh water (Table 2). It therefore follows that the proportion of non-chloride anions must also remain at the same level and, because these substances are continually entering the blood from the gut, they must be excreted in some way. Of the two possible pathways available for excretion, the renal route seems more likely than the extra-renal one because of the evidence derived from teleost material (Smith, 1930). In these animals, Ca^{2+}, Mg^{2+} and SO_4^{2-} ions which are taken up from the gut appear to be excreted in the urine. In the present experiments the quantity of urine produced was negligible, so that the non-chloride anions must either have been removed by the kidney and stored in any urine which may have been produced or they may have been extra-renally excreted.

THE BREAKDOWN OF MARINE OSMOREGULATION

An examination of Table 1 and Fig. 1 shows that the marine osmoregulatory powers of many fresh-run lamperns were failing. This failure can be attributed to changes in three of the main components of the marine osmoregulatory mechanism. The first two factors to be considered mainly affect the water balance of the animals.

Increase of the water permeability of the external surfaces

It has been argued already that an increase of water permeability may have taken place in animals which were found to be capable of osmoregulating in 50% sea water, and that this factor may be responsible in part for the breakdown of marine osmoregulation. Unfortunately, it has not been possible to assess water permeability in non-osmoregulating animals, so that this variable has had to be neglected in the analysis which follows.

The decrease in swallowing capacity

The problem of comparing the osmoregulatory ability of non-osmoregulating animals in terms of plasma freezing-point depression is complicated by unavoidable variations in the concentration of the external environment (Table 1). In osmoregulating animals it has been shown that an increase of environmental osmotic pressure from 33% sea water ($\Delta_t = 0{\cdot}660^\circ$ C.) to 50% sea water (mean $\Delta_t = 0{\cdot}970^\circ$ C.) brings about an increase of plasma freezing-point depression of 0·025° C. (Table 2). It thus seems that animals which are osmoregulating or partially osmoregulating are unlikely to be affected significantly by small variations in the external environment. In the series of animals showing lower plasma freezing-point depressions, small variations in sea-water concentration might be expected to have progressively more significant effects on plasma freezing-point depression as the animals become less independent of their environment. Even in the worst of these cases, however, the variation of the plasma must always be less than that of the environment, because the animals never reach isotonicity during the period of the experiments. For these reasons, variations in the concentration of the external environment have been neglected and osmoregulatory ability has been assumed to be directly related to plasma freezing-point depression.

The relationship between swallowing capacity and osmoregulatory ability (Fig. 2) is a complex one, because the plasma osmotic pressure also varies with the amount of extra-renal excretion which takes place at the same time. It is, therefore, only possible to consider the relationship in those cases where the extra-renal excretory powers can be assessed, that is, in those animals in which the number of chloride excretory cells could be classed either as 'many' or as 'absent'. In those animals containing the maximum number of excretory cells (Fig. 2, black triangles), the osmoregulatory ability decreases as the rate of swallowing decreases, and in osmoregulating animals the differences in swallowing rates are an expression of the effect of the differing water permeabilities of their external surfaces (Table 3). The animals without chloride excretory cells (open circles) show an increase in plasma osmotic pressure with an increased rate of swallowing. The explanation of this relationship is probably that the gut, being permeable to ions, allows these substances to enter the blood stream and, since the extra-renal excretory mechanism is lacking, the plasma osmotic pressure rises in proportion to the amount of water swallowed.

Although the cause of the reduction of swallowing capacity is uncertain, it is thought to be connected with degeneration of the alimentary tract. It is well known that in both the lampern and the sea lamprey the gut starts to atrophy when the animal commences its spawning migration (Keibel, 1927; Applegate, 1950). During the present studies, measurements of the gut diameter of maturing animals (March and April) showed that there was a great deal of variation in the pancreatic region, from almost complete closure (0·3 mm.) to maximum diameters of 2·3 mm. Some of these animals still swallowed a little sea water when immersed in 50% sea water, but there was only a weak correlation between cross-sectional area of the gut and swallowing capacity. This could have been due to the difficulties of assessing the cross-sectional area of the gut which, in these experiments, was only calculated approximately from the maximum and minimum diameters of the gut at its narrowest point, assuming an ellipsoid cross-section.

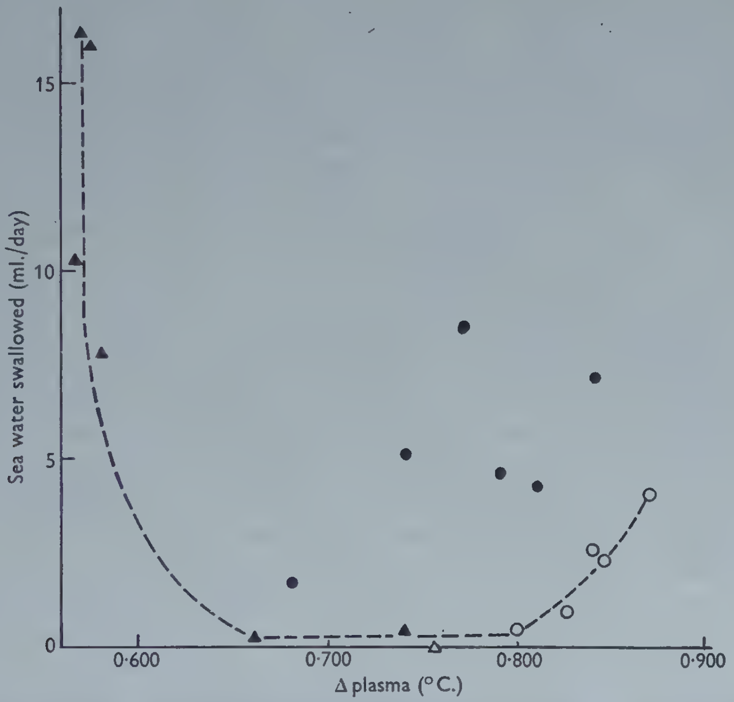

Fig. 2. The relationship between swallowing capacity, chloride excretory cell assessment and plasma freezing-point depression (Δ) in fresh-run lamperns immersed in 50% sea water. Chloride excretory cell assessment: ▲, many; ●, few; △, very few; ○, nil.

Decrease in extra-renal excretion

If those animals are considered which do not swallow sea water (Fig. 2), there appears to be a relationship between chloride excretory cell assessment and osmoregulatory capacity of the type which might be expected if extra-renal excretion was diminishing in some individuals. The pattern of behaviour of the animals containing 'few' excretory cells (black circles) is much more difficult to interpret, because these show the widest spread of variation in numbers of excretory cells, in osmotic response and in swallowing capacity. On the whole, however, they appear to support the general conclusions that a reduction in swallowing rate and/or a decrease in chloride excretory cell numbers results in a loss of osmoregulatory capacity.

DISCUSSION

Considered within the limits set by the present analysis, the marine mechanism of osmoregulation in the lamperns appears to be strikingly similar to that employed by marine teleosts (Smith, 1930; Keys, 1931*a*, 1931*b*, 1933). In these animals, the water and salts from swallowed sea water are absorbed selectively from a region of high concentration within the gut to one of lower concentration in the blood. Monovalent ions are absorbed faster than water and the divalent ions which remain in the gut are eliminated in the rectal fluid. Almost all of the excess monovalent ions are excreted extra-renally by tissues located in the gills which are capable of performing osmotic work, whilst the excess divalent ions which are absorbed are excreted in the scanty, but hypotonic urine. The end result of these processes is the continuous replacement of body water which is lost as a consequence of both the osmotic situation and the formation of urine. Apart from the renal elimination of divalent ions, where doubt remains because of the difficulties of analysis, the mechanism in the osmoregulating lamperns appears to follow the same pattern. The main point of difference between the lampern and the marine teleosts seems to be that the external surfaces of the former are very much more permeable to water. There may be at least two reasons why water permeability is very high in fresh-run lamperns. Bahr (1952) has shown that even newly metamorphosed and sexually immature adult lamperns begin to suffer in concentrations above 65% sea water and this, together with field observations, has led him to believe that the majority of adult lamperns spend their life in the reduced salinity found in estuarine waters. There is therefore little reason to expect most lamperns to develop the high resistance to osmotic loss which is so evident in marine teleosts. In addition to this, there is also evidence that the water permeability of the lampern changes during the course of its spawning migration. A comparison of the weight changes of fresh-run and maturing lamperns during the early stages of experiments involving immersion in dilute sea water, indicates that the water permeability of the external surfaces increases as the animals mature (Morris, 1956). This corresponds with the observation of Hardisty (1956), that mature animals show an increase in water content. Earlier work (Morris, 1956) also stressed the variability in water permeability in fresh-run animals and this is certainly borne out by the variable nature of extra-renal loss of water in the present experiments (Table 2). This variation may be the result of increased water permeability which would be expected to take place at different rates in some individuals at the beginning of the spawning migration. The low urine output recorded for osmoregulating animals indicates that they are suffering from water shortage and this also tends to support the view that the external surfaces have increased their permeability to water.

The observations on non-osmoregulating animals also confirm that the mechanism is teleost-like, since they give the opportunity of studying the effects of removal of parts of the mechanism without having to interfere with the animals surgically. Animals which have lost either the swallowing mechanism or the extra-renal excretory mechanism behave in ways which might be expected from a knowledge of the complete mechanism and thus confirm the osmoregulatory function of these processes. The relationship between osmoregulating ability and chloride excretory cell assessment is particularly important in view of the doubts expressed by Bevelander (1935, 1936) and Krogh (1939) concerning the excretory function of these cells. The present studies, together with those of Keys & Willmer (1932), Keys (1931*a*, 1931*b*), Copeland (1948), Liu (1942, 1944), Getman (1950) and Morris (1957), establish beyond reasonable doubt the excretory function of these structures in both the lampern and in marine teleosts.

Homer Smith has argued that the teleosts owe their peculiar marine osmoregulatory mechanism to their fresh-water ancestry. According to the view of Marshall & Smith (1930), the vertebrate kidney was developed originally as a means of dealing with the osmotic intake of water from this environment and, in consequence, its function was orientated toward water excretion and ion retention. When the teleosts invaded the seas, little change took place in kidney function initially in order to cope with changed osmotic circumstances, and the animals continued to produce a urine which was hypotonic to their blood, although diminished in quantity and augmented in ion content. This was made possible by the development of the swallowing habit combined with the extra-renal excretory mechanism described above (Smith, 1932, 1953). The ideas of Marshall and of Smith on the fresh-water origin of the vertebrate kidney have been questioned recently by Robertson (1954, 1957). Robertson finds that the earliest vertebrates were marine and argues for a marine origin of the glomerulus, since glomerular development does not appear to be proportional to the osmotic gradient in groups other than the Osteichthyes. These views do not, however, appear to conflict with Smith's hypothesis concerning the marine osmoregulating mechanism of the teleosts and there seems little doubt about the fresh-water origin of this group. Because of the remote relationship between lampreys and teleosts, the development of similar marine osmoregulatory mechanisms must have taken place independently and this could be attributed to parallel evolution brought about by similar osmotic circumstances during the past history of the two groups. It thus seems probable that the lampreys also developed in fresh water—a view which is strengthened by a consideration of their life history and migratory habits.

SUMMARY

1. Some fresh-run lamperns can osmoregulate in 50% sea water ($\Delta = 0\cdot970^\circ$ C.), where they can maintain plasma freezing-point depressions of about 0·57° C.

2. An analysis of the mechanism of osmoregulation in these animals shows that it is similar in many respects to that employed by marine teleosts. The lampern swallows sea water and absorbs a solution containing a high proportion of monovalent ions into its blood. It has been calculated that chloride is excreted by an extra-renal route, presumably by means of chloride excretory cells which have been discovered in the gills. The rate of extra-renal loss of water is high and the urine output is negligible.

3. Many fresh-run animals are unable to osmoregulate. In some cases the capacity to swallow sea water becomes reduced, whilst in others the number of chloride excretory cells is diminished—an indication that the extra-renal excretory mechanism is failing. Both of these mechanisms regress simultaneously in some animals.

I should like to thank Prof. E. J. W. Barrington for his valuable advice and criticism throughout the investigation.

REFERENCES

APPLEGATE, V. C. (1950). Natural history of the sea lamprey, *Petromyzon marinus*, in Michigan. United States Department of the Interior Special Scientific Report—Fisheries no. 55.

BAHR, K. (1952). Beitrage zur Biologie des Flussneunauges *Petromyzon fluviatilis* (Lebensraum u. Ernährung). *Zool. Jber.* (Syst.) **81**, 408.

BAKER, J. (1945). *Cytological technique.* Methuen Monographs on Biological Subjects. London: Methuen and Co. Ltd.

BARRINGTON, E. J. W. (1944). The supposed pancreatic organs of *Petromyzon fluviatilis* and *Myxine glutinosa*. *Quart. J. Micr. Sci.* **85**, 391.

BEVELANDER, G. (1935). A comparative study of the branchial epithelium in fish, with special reference to extra-renal excretion. *J. Morph.* **57**, 335.

BEVELANDER, G. (1936). Branchial glands in fishes. *J. Morph.* **59**, 335.

BURIAN, R. (1910). Funktion der Nierenglomeruli und Ultrafiltration. *Pflüg. Arch. ges. Physiol.* **136**, 741.

COPELAND, D. E. (1948). The cytology of the secretory cells of *Fundulus*. *J. Morph.* **82**, 201.

GETMAN, H. C. (1950). Adaptive changes in the chloride cells of *Anguilla rostrata*. *Biol. Bull., Woods Hole*, **99**, 439.

GRAFFLIN, H. L. (1931). Urine flow and diuresis in marine teleosts. *Amer. J. Physiol.* **47**, 602.

HARDISTY, M. W. (1956). Some aspects of osmotic regulation in lampreys. *J. Exp. Biol.* **33**, 431.

KEIBEL, F. (1927). Zur Entwicklungsgeschichte des Vorderdarmes und des Pankreas beim Bechneunauge (*L. planeri*) und beim Flussneunauge (*L. fluviatilis*). *Z. mikr.-anat. Forsch.* **8**, 409.

KEYS, A. (1931*a*). Heart-gill preparation of the eel and its perfusion for the study of a natural membrane *in situ*. *Z. vergl. Physiol.* **15**, 352.

KEYS, A. (1931*b*). Chloride and water secretion and absorption by the gills of the eel. *Z. vergl. Physiol.* **15**, 364.

KEYS, A. (1933). The mechanism of adaptation to varying salinity in the common eel and the general problem of osmotic regulation in fishes. *Proc. Roy. Soc.* B, **112**, 184.

KEYS, A. B. & WILLMER, E. N. (1932). 'Chloride secreting cells' in the gills of fishes, with special reference to the common eel. *J. Physiol.* **76**, 368.

KROGH, A. (1939). *Osmotic Regulation in Aquatic Animals.* Cambridge University Press.

LIU, C. K. (1942). Osmotic regulation and 'chloride secretory cells' in the Paradise fish, *Macropodus opercularis*. *Sinensia*, **13**, 15.

Liu, C. K. (1944). Sodium sulphate as an agent causing the development of 'chloride secretory' cells in *Macropodus. Nature, Lond.*, **153**, 252.

Marshall, E. K. & Smith, H. W. (1930). The glomerular development of the vertebrate kidney. *Biol. Bull., Woods Hole*, **59**, 135.

Moroney, M. J. (1951). *Facts from Figures.* Penguin Books.

Morris, R. (1956). The osmoregulatory ability of the lampern (*Lampetra fluviatilis* L.) in sea water during the course of its spawning migration. *J. Exp. Biol.* **33**, 235.

Morris, R. (1957). Some aspects of the structure and cytology of the gills of *Lampetra fluviatilis* L. *Quart. J. Micr. Sci.* **98**, 473.

Pitts, R. F. (1933). Urinary composition in marine fish. *J. Cell Comp. Physiol.* **4**, 389.

Reichert, E. T. & Brown, A. P. (1909). The differentiation and specificity of corresponding proteins and other vital substances in relation to zoological classification and organic evolution. *Carnegie Inst., Wash., Publ.* **116**, 30.

Robertson, J. D. (1954). The chemical composition of the blood of some aquatic chordates, including members of the Tunicata, Cyclostomata and Osteichthyes. *J. Exp. Biol.* **33**, 324.

Robertson, J. D. (1957). The habitat of the early vertebrates. *Biol. Rev.* **32**, 156.

Smith, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.* **93**, 480.

Smith, H. W. (1932). Water regulation and its evolution in fishes. *Quart. Rev. Biol.* **7**, 1.

Smith, H. W. (1953). *From Fish to Philosopher.* Boston: Little, Brown.

Thomas, O. L. (1948). A study of the spheroid system of sympathetic neurones with special reference to the problem of neuro-secretion. *Quart. J. Micr. Sci.* **89**, 333.

THE INTERNAL RHYTHM OF REPRODUCTION IN XEROPHILOUS BIRDS UNDER CONDITIONS OF ILLUMINATION AND DARKNESS

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(With Plate 10)

INTRODUCTION

Vaugien (1952, 1953) took young male Australian budgerygahs (*Melopsittacus undulatus*) shortly after they left the nest and divided them into three batches which were respectively placed: (1) under natural European daylengths; (2) in continuous light; and (3) in complete darkness. Samples of each group were killed for examination after 52 and 84 days. Spermatozoa had appeared within 52 days in all birds killed in each group (a total of 14 individuals). After 84 days the four survivors under continuous light contained only primary spermatocytes. Of the five birds under natural daylengths four held spermatozoa and the remaining individual only primary spermatocytes. Yet the six survivors in total darkness all contained spermatozoa.

These results have been widely quoted (e.g. Benoit & Assenmacher, 1953; Aschoff, 1955; Farner & Mewaldt, 1955). Vaugien concluded that total darkness seems to favour spermatogenesis in *Melopsittacus*, and that continuous light, on the contrary, appears to retard it. He was of the opinion that such results might find 'une interprétation dans les origines exotiques de cet oiseau' (Vaugien, 1953).

The budgerygah is a xerophilous form that in many areas breeds only after rainfall and irrespective of the season (Marshall, 1955). Therefore, although one could readily imagine that it might fail to respond to light, there is no factor in its environment to suggest that its sexual development could be enhanced by darkness.

Close examination of Vaugien's illustrations has provided an answer to the problem. The testes of birds kept in continuous light (primary spermatocytes only) are seen to have reached the 'regressed' condition that follows natural reproduction or gross laboratory photostimulation. In short, far from being less developed than the birds under natural illumination and total darkness, the constantly illuminated birds had far outstripped them. They had been driven right through one sexual cycle and had entered another. The organs with spermatocytes illustrated by Vaugien remarkably resemble those of xerophilous species that have been reactivated by unseasonal rainfall after a normal breeding season (Serventy & Marshall, 1957).

At the same time there is no suggestion of misinterpretation in regard to the rapid development of spermatozoa by *Melopsittacus* in total darkness. It was thought worthwhile therefore, to investigate the sexual reactions of a second, and unrelated, Australian xerophilous species to darkness and to varying degrees of illumination.

MATERIALS AND METHODS

The zebra (or chestnut-eared) finch (*Poephila castenotis* syn. *Taeniopygia castanotis*) resembles the budgerygah in occurring in extensive nomadic flocks that reproduce after rainfall irrespective of the annual cycle of the sun. When the young leave the nest the sexes are externally indistinguishable. The present study is based on forty-seven young birds of both sexes which were obtained while still in juvenile plumage. At this period the testes measure about 1 mm. in diameter (Pl. 10, fig. 1) and the largest ovarian oocytes are only about 360 μ in diameter. Beak-colour and plumage provide external indications of age. Juveniles of both sexes are drab, with almost black beaks. The beaks of both sexes, and the plumage of males, become progressively coloured until fully adult males possess a bright orange-red beak, chestnut ear-patches and white-spotted flanks. Adult females, too, possess an orange beak which is, however, slightly paler than that of the male. The female plumage remains comparatively drab.

The birds used were obtained from aviary suppliers and laboratory stocks. There is evidence (D. Morris, pers. comm.) that in general behaviour at least, such birds do not materially differ from those recently caught in the wild. The forty-seven experimental birds were kept in identical dietary, and almost identical housing, conditions at laboratory temperature, i.e. at an average 24 hr. temperature of 19° C. with negligible fluctuations. Nesting boxes and material were provided.

Experimental conditions were as follows:

I. Fifteen birds kept for 60 days in near darkness. (Faint indirect light was necessary to enable them to locate food, water and perches.)

II. Eight birds were kept under 6 hr. light per day for a period of 60 days. (This amount is of course far less than that which could be possibly experienced in their natural environment; see Table 1.)

III. Eight birds were kept under naturally decreasing photoperiods in London from 21 September to 21 November, i.e. from 12 hr. 19 min. to 8 hr. 33 min.

Table 1. *Daylengths to which* Poephila castenotis *is naturally subjected in the wild*

Geographical range	21 June	23 December
15° S.	11 hr. 7 min.	12 hr. 53 min.
35° S.	9 hr. 39 min.	14 hr. 21 min.

IV. Eight birds were kept during the same period as group III but under artificially increasing photoperiods equalling those of the corresponding period of spring.

V. Eight birds were kept under 15 hr. of light per day for 1 week, 17 hr. per day for a further week, and thereafter continuous light for a further 54 days, making a total of 68 days illumination.

At the conclusion of each experiment the birds were killed with coal gas. Descriptions were made of plumage and beak, and the gonads were dissected out and fixed in formol-calcium. One testis and half of each ovary were wax-embedded and stained with iron haemotoxylin for the identification of gametogenetic stages. Corresponding material was embedded in gelatine and coloured with Sudan black for the retention of interstitial and tubule lipids.

RESULTS

Group I (continuous near-darkness). Four males had the larger testis measuring 4 × 3 mm. Two others had testes enlarged to 3 × 2·5 mm. and 2·5 mm. in diameter. All these possessed bunched spermatozoa (Pl. 10, fig. 2). The oocytes of nine females had increased to only about 1 mm. in diameter. All birds had come into adult colour.

Group II. (6 hr. light daily). The larger testis of each of the three males measured between 4 mm. in diameter and 6 × 3 mm. long. All contained bunched spermatozoa. The five females possessed some oocytes that had enlarged to between 2 and 3 mm. in diameter. All birds in adult colour.

Group III (decreasing daylengths). The larger testis of each of the five males ranged between 4·5 mm. in diameter and 7 × 4 mm. long and contained bunched spermatozoa. The largest oocytes of the three females varied between 2 and 3 mm. in diameter. All birds in adult colour.

Group IV (increasing photoperiods). The larger testis of four males ranged between 4 mm. in diameter and 5.5 × 3 mm. long. All contained bunched spermatozoa. The largest oocytes in all four females had increased to between 2 and 3 mm. in diameter. All birds in adult colour.

Group V (almost continuous daylight). The four birds of each sex possessed gonads of the same average dimensions as those of group IV. The seminiferous tubules of each male contained bunched spermatozoa (Pl. 10, fig. 3). In one bird a tubule was seen already to be undergoing 'post-nuptial' steatogenesis (Pl. 10, fig. 4). All birds were in adult colour.

DISCUSSION

Vaugien's more striking assumption, i.e. that spermatogenesis is advanced more considerably in darkness than in light, has been examined and rejected on p. 666. His second remarkable conclusion appears to be confirmed. Certainly in the young of another xerophilous species, the zebra finch, complete spermatogenesis can take place within 60 days in the almost total absence of photostimulation.

At the same time, the zebra finch, like the budgerygah, is probably susceptible to photostimulation. Birds given 6 hr. daily illumination and those subjected both to increasing and decreasing photoperiods showed slightly greater testis development and strikingly greater ovarian development than the birds in the dark cage. All illuminated groups showed some interest in nesting materials. No nesting material was taken to the nest boxes in the dark cage.

The explanation of the above-described phenomena may be as follows:

Both the budgerygah and zebra finch are opportunist breeders, reproducing only when their generally dry environment irregularly offers conditions propitious for them to do so. If their cycles were rigidly controlled by photoperiodicity they would be eliminated by natural selection. If food and water remain plentiful, an internal rhythm of reproduction goes forward unchecked and the males produce spermatozoa even in darkness. The females require illumination before oocytes enlarge even to a limited extent. At the same time it is by no means sure that illumination has a direct effect on the reproductive processes of females. It may be that the light encourages behavioural reactions, including manipulation of nesting material, that affect the neuro-endocrine mechanisms and lead to gonad modification. It will be recalled that in all species yet investigated the male cycle far outstrips that of the female in the wild (Marshall, 1955).

Susceptibility to artificial illumination has already been proved in males but not females of the similarly xerophilous (even equatorial) weaver-finch (*Quelea quelea*) (Marshall & Disney, 1956). The cage experiments described above make it appear that the males at least of both zebra finch and budgerygah retain an ancestral capacity to respond to photostimulation. But under natural conditions any such tendency would be often over-ridden by external inhibitors that are significant to reproductive success.

The rapid production (within 60 days) of spermatozoa in the young of both parrot and finch is no doubt a physiological aspect of drought adaptation, i.e. it enables birds to reproduce quickly when the external environment is advantageous. Such speedy spermatogenesis occurred in warm cages well supplied with food and water. Under drought conditions external inhibitory factors would prevent rapid gametogenesis (Keast & Marshall, 1954; Serventy & Marshall, 1957).

SUMMARY

1. A report that total darkness is more effective than illumination in the production of spermatogenesis in young of the xerophilous budgerygah is shown to be due to a misinterpretation of results.

2. An internal rhythm of reproduction allows young male zebra finches (like young budgerygahs) to produce spermatozoa within 60 days in almost total darkness when provided with plenty of food and water at laboratory temperatures. Oocytes enlarge only slightly. Both sexes come into breeding dress in the dark.

3. Various conditions of illumination (including decreasing photoperiods) cause in both sexes greater gametogenetic activity than occurs in darkness.

4. Both budgerygah and zebra finch are xerophilous. They are opportunist breeders after rainfall, irrespective of season. Although they retain an ancestral capacity to respond to photostimulation, any such tendency under natural conditions could be over-ridden by external inhibitors.

5. The rapid production of spermatozoa (within 60 days) is in both parrot and finch a physiological aspect of drought adaptation enabling very young birds to reproduce quickly whenever environmental conditions are propitious.

REFERENCES

Aschoff, J. (1955). Jahresperiodik der Fortpflanzung bei Warmblütern. *Studium Generale*, **8**, 742.

Benoit, J. & Assenmacher, I. (1953). Action des facteurs externes et plus particulièrement du facteur lumineux sur l'activité sexuelle des oiseaux. *Reun. Endocr. fr.* **2**, 33.

Farner, D. S. & Mewaldt, L. R. (1955). Is increased activity or wakefulness an essential element in the mechanism of the photoperiodic responses of avian gonads? *Northw. Sci.* **29**, 53.

Keast, J. A. & Marshall, A. J. (1954). The influence of drought and rainfall on reproduction in Australian desert birds. *Proc. Zool. Soc.* **124**, 493–9.

Marshall, A. J. (1955). Reproduction in birds: the male. *Mem. Soc. Endocr.* **4**, 75–89.

Marshall, A. J. & Disney, H. J. de S. (1956). Photostimulation of an equatorial bird (*Quelea quelea*, Linnaeus). *Nature, Lond.*, **177**, 143–4.

Serventy, D. L. & Marshall, A. J. (1957). Breeding periodicity in Western Australian birds. *Emu*, **57**, 99–126.

Vaugien, L. (1952). Sur le comportement sexual singulier de la Peruch ondulee, maintenue à l'obscurité. *Comp. Acad. Sci.* **234**, 1489.

Vaugien, L. (1953). Sur l'apparition de la maturité sexuelle des jeunes perruches ondulées males soumises à diverses conditions d'éclairement: Le développement testiculaire est plus rapide dans l'obscurité complète. *Bull. Biol.* **87**, 274–86.

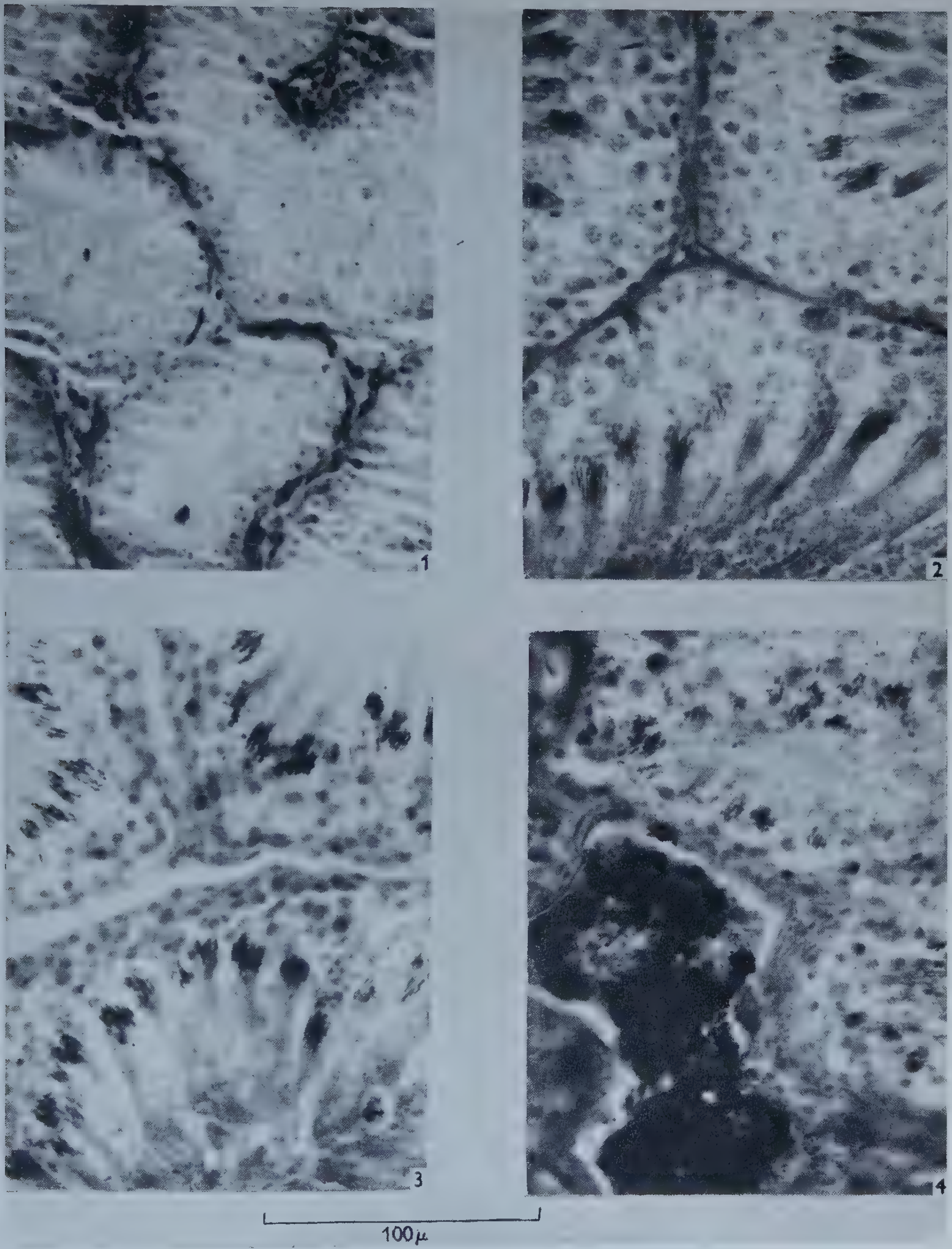
EXPLANATION OF PLATE

Fig. 1. Seminiferous tubules of young zebra finch at the age (40–45 days) used at the start of each experiment. There was in some birds already a minor spermatogenetic and colour development. Bouin fixation, wax embedding and iron haemotoxylin at 6μ.

Fig. 2. As fig. 1, but 100 days old after 60 days of almost total darkness. Technique as in fig. 1.

Fig. 3. As fig. 1, but 108 days old after 68 days of almost continuous illumination. Formalin fixation, gelatine embedding and Sudan black at 8μ.

Fig. 4. As Fig. 3; in this grossly over-stimulated bird one tubule has metamorphosed: 'postnuptial' steatogenesis has begun. Shortly the whole organ would collapse. Technique as in fig. 3.



MARSHALL AND SERVENTY—INTERNAL RHYTHM OF REPRODUCTION IN XEROPHILOUS BIRDS UNDER CONDITIONS OF ILLUMINATION AND DARKNESS

(*Facing p.* 670)

GAS SECRETION IN FISHES LACKING RETE MIRABILE

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(With Plate 11)

In most fishes with a closed swimbladder the gas secretion is confined to a special organ, the so-called red gland, which consists of a secretory epithelium supported by a counter-current capillary system, the rete mirabile. All the fishes investigated which possess this structure are capable of accumulating oxygen, and most of them also nitrogen, in the swimbladder against a pressure gradient. In other fishes the rete mirabile is lacking, and instead the secreting epithelium lines the swimbladder directly. Many fishes in this category apparently secrete only nitrogen.

Two lines of thought have been formulated in an attempt to explain the gas secretion. According to the oldest theory the glandular epithelium secretes acid into the blood, which through the Bohr effect dissociates oxygen from the haemoglobin. The primary oxygen release would accordingly take place in the blood of the gland capillaries, and from these oxygen would diffuse through the epithelium into the swimbladder. Because of seemingly insurmountable difficulties inherent in this theory a second view has been proposed, to the effect that the gas accumulation takes place by a process of cellular secretion whereby the glandular epithelium extracts the gases from the surrounding tissue and actively secretes them into the swimbladder.

The main difficulty with the acid-secretion theory becomes apparent when we consider gas accumulation against high pressures. The ultimate requirement must then be that the dissociation pressure of the oxygen produced by acidifying the haemoglobin would be as high, or higher, than the partial pressure of the oxygen found in the swimbladder. It has been shown by optical methods that this requirement is far from fulfilled in many deep sea fishes (Scholander & van Dam, 1954). Very likely the Bohr or Root effect plays a certain 'booster' role in the secretion process, especially at shallow depths, but it is incapable of explaining one of the most striking aspects of the phenomenon, namely, secretion against high pressures. In most fishes the oxygen accumulation is accompanied by accumulation of nitrogen and argon as well, and both of these inert gases may enter against considerable tension gradients, a situation left entirely unexplained by the acid-secretion theory.

If a closed container is filled with fully oxygenated blood and all of the oxygen is released from the haemoglobin by, for instance, ferricyanide, the tension developed is simply the ratio between the oxygen capacity and the solubility coefficient, and may amount to some 2–3 atmospheres oxygen pressure in fish blood. In the presence of a rete mirabile counter-current system such a primary pressure effect, if caused by a non-diffusible agent, would easily be multiplied by a factor of several hundred, but without a rete no more pressure could be produced in the blood than would correspond to the above ratio. This makes it attractive to consider what gas pressures may develop in a swimbladder where the rete mirabile system is lacking.

In the present investigation we have accordingly studied the blood of some salmonid fishes lacking a rete mirabile and yet capable of secreting oxygen and nitrogen against considerable pressure. It will be seen that our data cannot be explained in terms of the acid-secretion theory, but point rather to an active cellular process.

MATERIAL AND METHODS

The fishes for this investigation, *Coregonus lavaretus* and *Salmo alpinus*, were obtained in the Ransfjord lake in Norway and *Coregonus acronius* in the Bodensee in Germany. They were caught by gill nets at various depths, ranging from 2 to 70 m. Gas samples were taken from live fish immediately the net reached the surface. The gas was analysed in a syringe analyser (Scholander, van Dam, Claff & Kanwisher, 1955).

Gas samples from the whitefish *C. acronius* were taken in syringes sealed with a saturated LiCl solution, and analysed by mass spectrometer for A/N_2 and $^{14}N/^{15}N$ ratios.

Blood samples were drawn from live fish by heart puncture, and were analysed for oxygen and nitrogen capacity in a Scholander–Roughton syringe. The fish were kept breathing in an irrigated rubber mask to avoid lactic acid formation. The procedure for oxygen analysis in fish blood (Scholander & van Dam, 1956) was modified in two respects: (1) the sequence of acid sulphate and potassium ferricyanide was reversed; and (2) the bicarbonate content of the potassium ferricyanide solution was doubled.

The nitrogen was analysed according to a modification of the above procedure. Three times the amount of blood was used and the oxygen was absorbed by the Fieser reagent (Edwards, Scholander & Roughton, 1943).

The depth of buoyancy was determined by the gas volume in the swimbladder divided by the submerged weight of the fish, according to the method of Saunders (1953).

EVIDENCE FOR LACK OF COUNTER-CURRENT SYSTEM

In whitefish and char the epithelia lining the swimbladder are very similar, consisting of cylindrical cells in the anterior part and cubical cells in the posterior part (Pl. 11). Gas secretion is presumably associated with the cylindrical epithelium. Beneath this layer the tissue is only sparsely supplied with capillaries which do not form any discernible rete structure. The lack of a functioning rete was independently indicated by the analyses of the nitrogen isotope ratio $^{14}N/^{15}N$ in the gas obtained from the Bodensee whitefish caught at 65 m. depth. The ratio was the same as for atmospheric nitrogen to within ± 0·3 %. This lack of separation of isotopes may be taken to indicate that a counter-current diffusion process is absent.

OXYGEN SECRETION WITHOUT A RETE MIRABILE

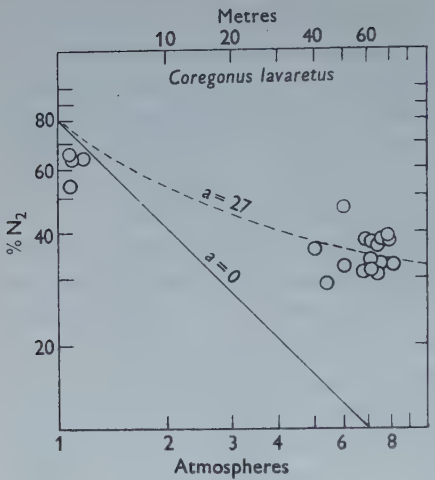
One of the salmonids investigated, *C. lavaretus*, unlike other whitefish, was found to accumulate considerable amounts of oxygen in the swimbladder. The oxygen fraction increased with depth (Text-fig. 1), as it does in deep-sea physoclist fishes (Scholander & van Dam, 1953).

It is a fair assumption that the secretion pressure must equal the buoyancy depth, i.e. the hydrostatic pressure at which the fish is in neutral buoyancy. Unfortunately *C. lavaretus* suffered a conspicuous loss of gas during its ascent to the surface, with the result that estimates of its buoyancy depth were too low (Text-fig. 2). One may safely say, however, that the secretion pressure must lie somewhere between the depth of buoyancy and the depth of catch, i.e. between 3·5 and 5 atmospheres (Table 1).

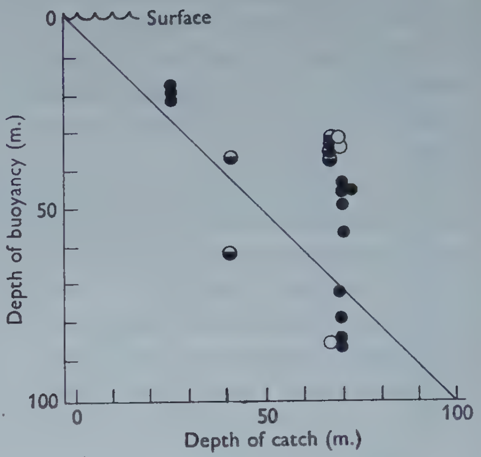
The oxygen capacity of the fish blood at lake temperature (4° C.) was found to be near 8 vol. % (Table 2). It has never been observed that more than 60% of any fish blood could be inactivated by acidification (Scholander & van Dam, 1954), and we can therefore expect no more than 4·8 vol. % oxygen to be released in the whitefish blood. The solubility coefficient for oxygen in water at 4° C. is near 4·4 vol. % and somewhat less in blood, and hence the maximum oxygen pressure which could be developed in the blood would be 4·8/4·4 = 1·1 atmospheres, which is one-quarter or at best one-third of the observed oxygen tension in the swimbladder. We may therefore conclude that a primary one-step oxygen release in the blood capillaries supplying the swimbladder epithelium cannot produce an oxygen tension as high as exists in the swimbladder of the fish.

SECRETION OF NITROGEN AND ARGON

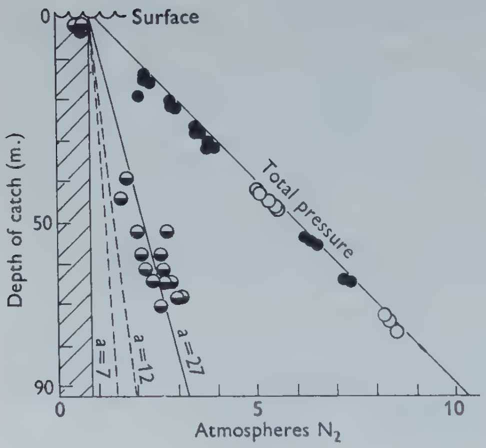
The partial pressure and fractional concentration of nitrogen in the swimbladder gas of the Bodensee whitefish and the char is shown in Text-fig. 3. It is clear that the nitrogen tension in both these fishes could rise to some 7–8 atmospheres pressure as compared to 0·8 atmosphere in the lake water, and the question arises: how does this accumulation of nitrogen against high pressure come about? One possibility would be a liberation of nitrogen from some compound in the blood effected by a secretory product from the epithelial cells. The released nitrogen would then diffuse into the swimbladder, i.e. a process in full analogy with the acid-secretion theory of oxygen. Such a chemical release of nitrogen from the blood is unlikely, because the A/N_2 ratio found in the swimbladder, although low (54–70% of that of air), was not nearly as low as would be expected if the argon were left behind by a chemical secretion of nitrogen (Koch, 1934; Scholander, van Dam & Enns, 1956).



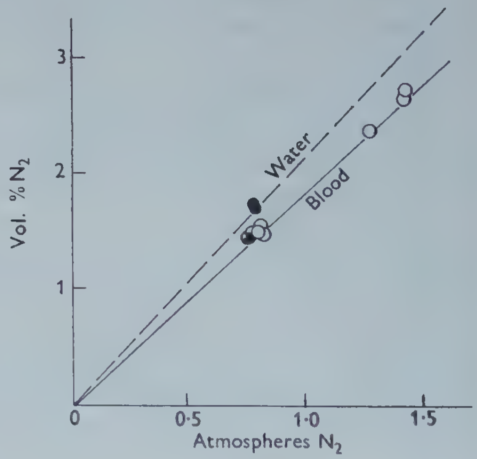
Text-fig. 1



Text-fig. 2



Text-fig. 3



Text-fig. 4

Text-fig. 1. Percentage of nitrogen found in the swimbladder of *Coregonus lavaretus* at various depths. The diagonal line represents the percentage of nitrogen which at any given depth would give a partial pressure of nitrogen of 0·8 atmosphere. *a*, the nitrogen as percentage of the total gas secreted.

Text-fig. 2. Depth of buoyancy of *Coregonus acronius*, *C. lavaretus*, and *Salmo alpinus* in relation to depth of catch. The diagonal is the line of perfect buoyancy, i.e. where depth of buoyancy equals depth of catch. ●, *C. acronius*; ◒, *C. lavaretus*; ○, *S. alpinus*.

Text-fig. 3. Nitrogen tension in the swimbladder of *Coregonus acronius*, *C. lavaretus* and *Salmo alpinus* in relation to depth. *a*, the nitrogen as percentage of the total gas secreted, 7 for *Macrourus*, 12 for *Sebastes*, and 27 *for Coregonus lavaretus*. Cross-hatched area represents nitrogen tension in the lake water. ●, *C. acronius*; ◒, *C. lavaretus*; ○, *S. alpinus*; ---, physoclist fish.

Text-fig. 4. Nitrogen content in the blood of *Salmo alpinus* and *Coregonus lavaretus* equilibrated at various pressures of nitrogen at 3·8° C. ●, Six water analyses performed by the same method, which agree with the drawn line for the solubility of nitrogen in water; ◒, *C. lavaretus*; ○ *S. alpinus*.

Table 1. *Partial pressure of oxygen and nitrogen in the swimbladder of whitefish* (Coregonus lavaretus) *calculated from depth of catch and depth of buoyancy*

No. of specimens	Depth of catch (mean values) (m.) A	Depth of buoyancy (mean values) (m.) B	pO_2 atm. (mean values)		pN_2 atm. (mean values)	
			A	B	A	B
10	1–2	0	0·4	0·4	0·7	0·7
8	40	38	3·5	3·1	1·7	1·6
12	70	45	5·0	3·5	2·9	1·9

Table 2. *Oxygen capacity of haemoglobin in blood of salmonids (blood adjusted to pH* 7·8 *and saturated with air. Physically dissolved oxygen subtracted)*

Species	No. of specimens	Temp. (° C.)	Vol. %	Average
Coregonus lavaretus	9	3·8	6·6–9·4	8·0
Coregonus acronius	4	15·0	4·6–5·0	4·8
Salmo alpinus	2	3·7	6·4–9·0	7·7
Salmo alpinus	3	2·8	8·7–9·9	9·3

If, nevertheless, such a process took place it would have to be capable of releasing up to 7–8 times the normally dissolved nitrogen in order to account for the observed nitrogen tension in the swimbladder. It would seem that such a large amount of dissociable nitrogen would be easy to detect. Blood from char and whitefish was therefore equilibrated with nitrogen at various pressures at 4° C., and the total amount of molecular nitrogen given off by acid ferricyanide and vacuum was determined gasometrically. From this it was established that the amount of nitrogen present in the blood was no greater than that physically dissolved, and that the nitrogen content followed Henry's law exactly (Text-fig. 4).

We may conclude that there is no positive evidence for chemical release of nitrogen into the blood, but indeed very strong evidence against it.

CONCLUSIONS

We have shown that the maximum oxygen pressure which may be obtained by acidifying fish blood is much less than the observed oxygen tension in the swimbladder. We have been unable to show that our fishes possess means of adding in any way to the molecular nitrogen which is physically dissolved in the blood. Inasmuch as they lack a counter-current multiplying system in the form of a rete mirabile we must conclude that the principal pressure build-up of oxygen and nitrogen does not take place in the blood but is a function of the epithelium lining the swimbladder. This agrees with the findings that the dissociation pressure of oxygen obtained by acidifying blood from various deep-sea fishes frequently falls short of the observed oxygen tension in the swimbladder (Scholander & van Dam, 1954).

SUMMARY

1. Gas secretion has been studied in three species of salmonid fishes, one of which accumulates both nitrogen and oxygen against considerable gradients, while the other two accumulate only nitrogen. None of these three species possesses a counter-current system (rete mirabile) which could step up a slight primary pressure effect.

2. Neither the nitrogen nor the oxygen capacity of the blood is large enough to account for the tensions found in the swimbladder by a simple one-step diffusion process.

3. One is forced therefore to consider a mechanism for active transport of these gases by the cells lining the swimbladder.

The present work was made possible by the support of Det Vitenskapelige Forskningsfond av 1919 and Nansenfondet. We are greatly indebted to Dr W. Nümann and his staff for their helpful arrangements for our work at the Institut für Seenforschung und Seenbewirtschaftung, Langenargen am Bodensee, and to Mr O. J. Berger, Jevnaker, for giving us the utmost co-operation in securing the material from the Norwegian lake.

REFERENCES

EDWARDS, G. A., SCHOLANDER, P. F. & ROUGHTON, F. J. W. (1943). Micro gasometric estimation of the blood gases. III. Nitrogen. *J. Biol. Chem.* **148**, 565–71.

KOCH, H. (1934). L'émission de gaz dans le vésicule gazeuse des poissons. *Rev. Quest. sci.* (ser. 4), **26**, 385–410.

SAUNDERS, R. L. (1953). The swimbladder gas content of some freshwater fish with particular reference to the physostomes. *Canad. J. Zool.* **31**, 547–60.

SCHOLANDER, P. F. & VAN DAM, L. (1953). Composition of the swimbladder gas in deep sea fishes. *Biol. Bull., Woods Hole*, **104**, 75–86.

SCHOLANDER, P. F. & VAN DAM, L. (1954). Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol. Bull., Woods Hole*, **107**, 247–59.

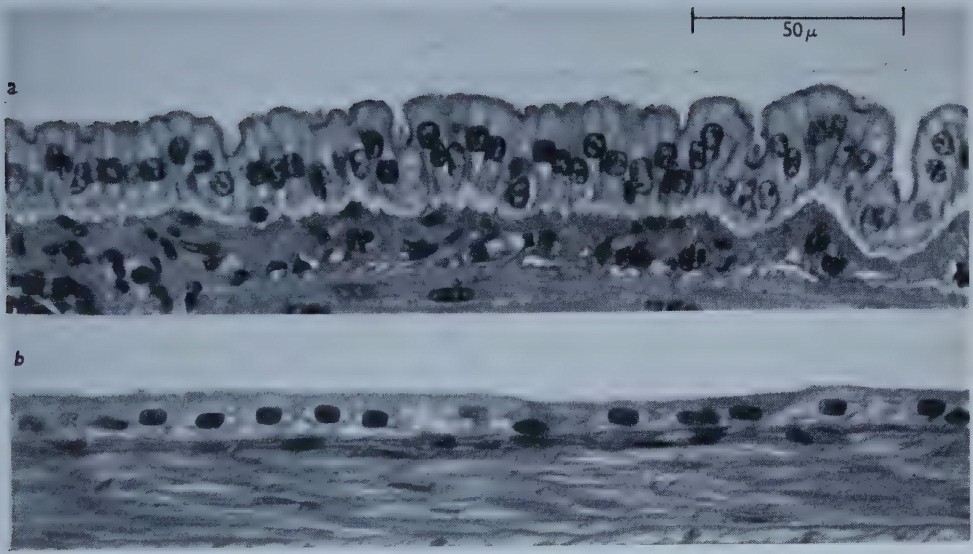
SCHOLANDER, P. F. & VAN DAM, L. (1956). Micro gasometric determination of oxygen in fish blood. *J. Cell. Comp. Physiol.* **48**, 529–32.

SCHOLANDER, P. F., VAN DAM, L., CLAFF, C. L. & KANWISHER, J. W. (1955). Micro gasometric determination of dissolved oxygen and nitrogen. *Biol. Bull., Woods Hole*, **109**, 328–34.

SCHOLANDER, P. F., VAN DAM, L. & ENNS, T. (1956). Nitrogen secretion in the swimbladder of whitefish. *Science*, **123**, 59–60.

EXPLANATION OF PLATE

The epithelium lining the swimbladder of *Coregonus lavaretus*; *a*, cylindrical cells of the anterior part; *b*, cubical cells of the posterior part.



SUNDNES, ENNS AND SCHOLANDER—GAS SECRETION IN FISHES LACKING RETE MIRABILE

COMPARISON OF DEHYDRATION AND HYDRATION OF TWO GENERA OF FROGS (*HELEIOPORUS* AND *NEOBATRACHUS*) THAT LIVE IN AREAS OF VARYING ARIDITY

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INTRODUCTION

Water conservation presents special problems to terrestrial Amphibia because: (*a*) evaporation from the naked skin is largely uncontrolled, and (*b*) no frog has been described which can secrete hypertonic urine.

Since frogs of different kinds do inhabit a variety of ecological situations with irregular rainfall and high rates of evaporation, the mechanism of survival has been the focus of attention and speculation.

An analysis of this problem demands answers to the following physiological questions:

(i) Rate of dehydration in dry environments?
(ii) Degree and duration of dehydration consistent with survival?
(iii) Rate of hydration when desiccated animals are exposed to moisture?

If the answers to (i), (ii) and (iii) indicate that different species have different capabilities, the question arises as to whether the physiological responses correlate with habitat. If this is not so one must go beyond physiology and see whether natural history observations indicate the existence of behavioural responses which allow the animal to avoid the stress.

With regard to the three physiological points enumerated above there is scant evidence.

(i) Thorson (1955), working on a variety of species from different environments, showed that water loss was always the same per unit area of skin.

(ii) Thorson & Svihla (1943), working with several species, showed that there was correlation between ability to withstand desiccation and degree of aridity of the habitat.

(iii) Ewer (1952) showed that water uptake after desiccation was greater in terrestrial species of *Bufo* than in the aquatic *Xenopus*. Thorson (1955) compared water uptake after desiccation in the ecologically dissimilar species *Rana pipiens*, *R. clamatans* and *Scaphiopus hammondii* but could find no correlation between rate of water uptake and aridity of the habitat.

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If meanwhile we accept, admittedly on meagre evidence, that the only physiological differences between different frog species are (*a*) ability to withstand a high degree of desiccation, and (*b*) rate of water uptake when presented with water, then the question of mechanism arises. There is no data with regard to (*a*), but there is abundant evidence (Jørgensen, 1950; Ewer, 1952) that the posterior pituitary influences both uptake of water and urinary flow while Ewer (1952) has shown that forms which hydrate quickly exhibit a greater response to injection of posterior pituitary extracts than others which hydrate slowly.

The frogs of Western Australia, because of the number of species and the variety of habitats, offer special opportunities for a more complete analysis of mechanism in relation to environment than has hitherto been possible. In the present paper two endemic Australian genera *Neobatrachus* and *Heleioporus* are considered, *Neobatrachus* with four species and *Heleioporus* with five. It has thus been possible to make interspecies as well as intergeneric comparisons in relation to the considerations outlined above.

METHODS

Collection and preliminary treatment. Jørgensen, (1950) has pointed out the difference in water uptake that may occur at different times of the year. All the species used in these experiments were collected between February and May, i.e. summer and early autumn, when rain and temperature conditions were favourable for breeding, and so they were all presumed to be in a similar physiological condition. Weights of the various species were measured at the beginning of the experiments and are set out in Table 1.

Table 1. *Size of all the species of* Neobatrachus *and* Heleioporus *used in the experiments*

Species	Mean body weight (B.W.) ± standard deviation (g.)	Surface area (cm.^{2}/g./B.W.)
N. pelobatoides (19)	6·0 ± 1·22	3·30
N. centralis (11)	12·1 ± 4·05	2·61
N. sutor (6)	6·7 ± 1·33	3·18
N. wilsmorei (6)	27·1 ± 3·69	2·00
H. psamophilus (13)	10·2 ± 2·05	2·78
H. inornatus (14)	18·9 ± 6·51	2·26
H. eyrei (17)	19·7 ± 6·26	2·22
H. australiacus (6)	34·8 ± 12·50	1·88
H. albopunctatus (13)	49·5 ± 20·92	1·63

In parentheses: number of animals.

The animals were predominantly males, though a few non-gravid females were included in the experiments. There was no evidence of any statistically significant difference ($P > 0·05$) in water-uptake response between the sexes.

The frogs, kept in beakers containing tap water in a constant temperature room at 21° ± 1° C., were not fed, and were allowed 3–4 days in the beakers containing tap water before experiments were commenced. This minimized any chance of sudden weight changes due to defaecation.

The surface area of the frogs was calculated using the formula of Rey (1937).

Ability to withstand dehydration. After the urinary bladder of each animal had been emptied by catheterization, dehydration was carried out in wire cages at 21° ± 1° C. and 55% relative humidity. As the survival limits of water loss were approached the frogs were weighed at hourly intervals. The last measurement before death was taken as the limit of water loss that could be withstood. Owing to shortage of animals no systematic attempts were made to see if recovery from this penultimate degree of dehydration was possible. A few experiments on each species suggested, however, that recovery was frequently possible.

Dehydration and water uptake. Animals were dehydrated as described above. When about 25% of the body weight had been lost by evaporation, they were placed in tap water and weight increases were measured at half hourly intervals. A weight loss of 25% was found to be always consistent with survival. Water uptake in the first hour after catheterization was found to give a representative picture, and was used as the parameter for comparison between species.

Water uptake with posterior pituitary extracts. After the bladder had been emptied the frogs were weighed to 0·1 g. A solution of Pitocin (Park, Davis and Co.) made up to appropriate strength with distilled water, was injected into a dorsal lymph sac. This had been previously found to be the most active posterior pituitary fraction affecting the two genera under observation. The injected volume amounted to 1% of the body weight at a dosage of 1 u./100 g. The animals were placed in tap water, catheterized 3 hr. later and weighed, increase in weight being taken to indicate water retained. Obviously, what was being measured was not the absolute water uptake through the skin but that portion that was retained due to an anti-diuresis. This would appear to be the important part of the whole response that would effect the survival of an animal with an intermittent water supply in nature.

RESULTS

No data are available on rate of water loss, the work being confined to the following:

Degree of dehydration consistent with survival

All species died after losing 40–45% of their body weight. An analysis of variance failed to show up any differences in the ability to withstand desiccation (Table 2) between any of the species of the two genera tested.

Water uptake after dehydration

The results are given in Table 3. An analysis of variance showed that there were no significant differences between the different species of *Heleioporus*. However, a similar analysis of the results from *Neobatrachus* indicated there were highly significant differences between the species. *N. centralis* took up water more rapidly than *N. pelobatoides*, while *N. sutor* and *N. wilsmorei* each took up water more

Table 2. *Ability of frogs of the genera* Neobatrachus *and* Heleioporus *to withstand desiccation*

Species	% loss of body weight at death	Species	% loss of body weight at death
H. psamophilus	41·3 (13)	*N. pelobatoides*	38·8 (17)
H. inornatus	40·1 (12)	*N. centralis*	41·7 (5)
H. eyrei	40·7 (18)	*N. sutor*	44·1 (5)
H. australiacus	38·9 (6)	*N. wilsmorei*	43·9 (6)

Error variance from the analysis of variance 1201·2747 (g. 100 g.)^2 on 83 D.F., variance ratio 1·84 on 8 and 83 D.F. which is not significant.
Results are the means of the numbers in parentheses.

Table 3. *Water uptake in species of the genera* Neobatrachus *and* Heleioporus *after dehydration*

	Water uptake (mg./cm.^{2}/hr.)		Water uptake (mg./cm.^{2}/hr.)	Significance of differences
H. psamophilus	44·0 (12)	(*a*) *N. pelobatoides*	33·3 (20)	(*a*)–(*b*) $P<0·01$
H. inornatus	55·0 (13)	(*b*) *N. centralis*	55·7 (11)	(*b*)–(*c*) $P<0·01$
H. eyrei	52·5 (18)	(*c*) *N. sutor*	84·8 (6)	(*c*)–(*d*) N.S.
H. australiacus	60·0 (6)	(*d*) *N. wilsmorei*	99·4 (6)	—
H. albopunctatus	57·7 (13)			

Error variance from analysis of variance: (i) *Heleioporus* 286·3448 (mg./cm.^{2}/hr.)^2 on 57 D.F. variance ratio 1·40 on 57 and 4 D.F. which is not significant; (ii) *Neobatrachus* 393·4775 (mg./cm.^{2}/hr.)^2 on 39 D.F.variance ratio 22·58 on 39 and 3 D.F. $P<0·001$.
Results are the means of the numbers in parentheses.

rapidly than *N. centralis*. The difference in rate of water uptake between *N. sutor* and *N. wilsmorei* was not found to be statistically significant. The water uptake in the four species of *Neobatrachus* shows close correlation with the increasing aridity of the environment measured not by the annual rainfall but by the number of days in a year in which rain falls. Thus the species occupying regions with fewer days on which rain falls have the greatest rate of water uptake.

Small frogs have a greater surface area relative to body weight than large frogs (Table 1), and if water uptake per unit surface area is the same, small frogs would be expected to gain water more rapidly per unit body weight than large frogs. The possibility cannot be ruled out that this may place the larger frogs at a disadvantage and selective factors may be expected to produce a greater water uptake per unit surface area in the larger animals. It is therefore surprising that *N. sutor* and *N. wilsmorei*, which differ markedly in size and occupy the same geographical territory, are the two species that take up water most rapidly. Furthermore, *N. pelobatoides* and *N. sutor*, which are similar in size, take up water at markedly different rates. From Table 1 it can be seen that *N. wilsmorei* has only 61 % the surface area per unit body weight of *N. pelobatoides*. *N. wilsmorei* takes up water at a rate of 19·75 ± 0·62 (S.E.) g./100 g./hr., while *N. pelobatoides* takes it up at

10·80 ± 0·507 (S.E.) g./100 g./hr. The difference is highly significant ($t = 4{\cdot}237$, $P < 0{\cdot}001$). *N. wilsmorei* thus takes up water faster than *N. pelobatoides* per unit body weight as well as per unit surface.

Effects of posterior pituitary extracts

In view of the demonstration by Brunn (1921) of an increase in water uptake by frogs injected with posterior pituitary extracts and subsequent repeated confirmation of these observations (see Heller, 1945, 1950; Sawyer, 1956) it was of interest to see if differences observed in the rate of water uptake after dehydration were also seen after the injection of posterior pituitary extracts. The results are given in Table 4. In the genus *Heleioporus* no statistically significant differences in response were observed to injection of Pitocin. In the genus *Neobatrachus* differences similar to those observed after dehydration were seen.

Table 4. *Water uptake in species of the genera* Neobatrachus *and* Heleioporus *injected with* 1 *u. Pitocin*/100 *g. body weight*

	Water uptake (mg./cm.^{2}/3 hr.)		Water uptake (mg./cm.^{2}/3 hr.)	Significance of differences
H. psamophilus	51·4 (12)	(*a*) *N. pelobatoides*	31·7 (20)	(*a*)–(*b*) $P < 0{\cdot}01$
H. inornatus	40·1 (14)	(*b*) *N. centralis*	52·9 (11)	(*b*)–(*c*) N.S.
H. eyrei	44·1 (17)	(*c*) *N. sutor*	56·6 (6)	(*c*)–(*d*) $P < 0{\cdot}01$
H. australiacus	58·8 (6)	(*d*) *N. wilsmorei*	98·6 (6)	—
H. albopunctatus	46·2 (13)			

Error variance from analysis of variance (i) *Heleioporus* 195·3298 (mg./cm.^{2}/3 hr.)^2 on 58 D.F. variance ratio 2·43 on 58 and 4 D.F. which is not significant. (ii) *Neobatrachus* 309·6640 (mg./cm.^{2}/3 hr.)^2 variance ratio 22·48 on 38 and 3 D.F. $P < 0{\cdot}001$.

Results are the means of the numbers in parentheses.

Because of the above findings, that desert species rehydrate more rapidly than non-desert forms, it was decided to make a comparison of two populations of *N. pelobatoides* from geographical extremes of the species range. A sample of eleven *N. pelobatoides* from the eastern limit of the range was compared with the *N. pelobatoides* mentioned in Table 4 which were from the western extreme of the range. The water uptake of the eastern animals when injected with 1 u./100 g. Pitocin was 28·1 ± 3·01 mg./cm.^{2}/3 hr. is very similar to the water uptake of the western animals (Table 4) injected with the same dose of Pitocin. A comparison of the animals' ability to take up water after dehydration unfortunately could not be made as these animals were particularly sensitive to captivity and died.

DISCUSSION

Fig. 1 shows the geographical distribution of the two genera in Western Australia. Although a large territory is common to both, the results given above show that the two genera in no way parallel each other except in an ability to withstand desiccation. This apparent contradication can be resolved by considering the ecology of the genera.

All species of the *Heleioporus* are burrowers and usually frequent sandy or friable soil. During summer, burrows reach the damp subsoil and some specimens of *H. albopunctatus* have been dug from burrows 33 in. deep. The animal buries itself but does not remove the soil from the burrow, which is thus effectively sealed. Of the *Heleioporus* species the range of *H. albopunctatus* extends farthest into the region of low and uncertain rainfall; this species aestivates, during summer, in a micro-environment very similar to that occupied by all other species of the genus.

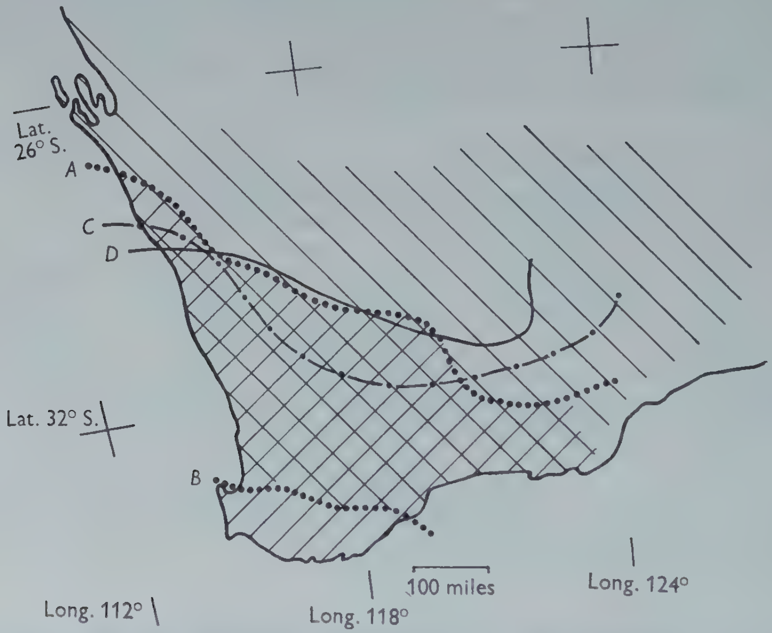

Fig. 1. Geographical distribution of (*a*) *Neobatrachus*, \\\\\\; (*b*) *Heleioporus*, ///. *A*, 60 wet days a year; *B*, 120 wet days a year; *C*, south-western boundary of *N. sutor*; *D*, south-western boundary of *N. wilsmorei*.

The genus *Neobatrachus* frequents clayey soils in which deep burrows are not possible. These burrows do not give the humid micro-environment provided by the burrows of *Heleioporus*. The water uptake in the various species of this genus closely follows the dryness of the habitat. Thus *Neobatrachus pelobatoides* is common between the isopleths of 120 wet days/year and 60 wet days/year (wet day defined as when 0·01 in. of rain falls in a day). In the drier part of the range *N. pelobatoides* is restricted to localized habitats adjacent to large rock outcrops, where additional run-off ameliorates the aridity. At the other extreme, *N. sutor* and *N. wilsmorei* persist into regions where there are less than 40 wet days/year. The inland penetration of these two species is not known, but as *N. wilsmorei* has the lowest surface area per unit body weight and the fastest water uptake per unit surface area it might be expected that this species would persist in more arid areas than *N. sutor*.

Rate of water uptake would be under positive selective pressure if water supplies became available only sporadically and for limited periods. Dew may be present on cold mornings in inland areas occupied by *Neobatrachus*, but at these low temperatures the animals are inactive. The sporadic rains indicated by the number of wet days (see above), combined with the rapid disappearance of surface water, would seem to define the conditions under which rapid uptake of water would be an advantage.

Thorson (1955) found that *Scaphiopus hammondii*, from drier areas than any other species tested, actually took up water more slowly. As shown above with *Heleioporus*, rate of water uptake need not necessarily correlate with aridity of the environment if a behaviour pattern is present by which risk of serious desiccation is avoided.

The species tested in the present experiments showed no difference in ability to withstand desiccation. All fall about midway in the pattern described by Thorson & Svihla (1943) being inferior to *Scaphiopus*, but far more able to withstand water loss than the aquatic and semi-aquatic species of *Rana* used by Thorson & Svilha.

The response of water uptake after injection of Pitocin in the genus *Neobatrachus* was paralleled by the rate of water uptake after dehydration. This observation, together with the demonstration of active posterior pituitary principles in frogs and toads (see Jørgensen, 1950), seems to indicate that water uptake in dehydrated frogs is potentiated by release of endogenous posterior pituitary hormone. The objection to this view is the observation of Jørgensen, Wingstrand & Rosenkilde (1956) that neurohypophysectomized *Bufo bufo* take up water as rapidly after dehydration as intact animals. It is felt, however, that before these experiments are taken to invalidate this conclusion the experiments of Jørgensen *et al.* (1956) should be repeated on a genus such as *Neobatrachus* which, because of the species differences in rate of water uptake, offer comparative material more likely to show significant differences between intact and neurohypophysectomized animals if in fact the posterior pituitary is physiologically significant in this respect.

SUMMARY

1. Ability to take up water in response to dehydration and injection of posterior pituitary extract was measured in two genera of frogs, *Heleioporus* and *Neobatrachus*, that live in areas of varying aridity in south-west Australia.

2. Species of *Neobatrachus* from dry areas took up water more rapidly than those from less dry ones. No such correlation could be seen in the species of *Heleioporus*.

3. No differences could be seen in the ability of any of the animals to withstand desiccation.

4. These findings are discussed in relation to the animals' behaviour in its natural environment.

This work was carried out with the aid of research grants from the University of Western Australia. Mr N. Stenhouse of C.S.I.R.O. kindly performed a statistical analysis of the results.

REFERENCES

BRUNN, F. (1921). Beitrag zur Kenntnis der Wirkung von Hypophysenextrakt auf den Wasserhaushalt des Frosches. *Z. ges. exp. med.* **25**, 170–5.

EWER, R. F. (1952). The effects of posterior pituitary extracts on water balance in *Bufo carens* and *Xenopus laevis*, together with some general considerations of anuran water economy. *J. Exp. Biol.* **29**, 429–39.

HELLER, H. (1945). The effect of neurohypophyseal extracts on the water balance of lower vertebrates. *Biol. Rev.* **20**, 147–57.

HELLER, H. (1950). The comparative physiology of the neurohypophysis. *Experientia*, **6**, 368–76.

JØRGENSEN, C. B. (1950). The amphibian water economy, with special regard to the effect of neurohypophyseal extracts. *Acta. physiol. scand.* **22**, suppl. 78.

JØRGENSEN, C. B., WINGSTRAND, K. G. & ROSENKILDE, P. (1956). Neurohypophysis and water metabolism in the toad, *Bufo bufo* (L.). *Endocrinology*, **59**, 601–10.

REY, P. (1937). Recherches expérimentales sur l'économie de l'eau chez les Batraciens. Dissertation, Paris.

SAWYER, W. H. (1956). The hormonal control of water and salt-electrolyte metabolism with special reference to the amphibia. *Mem. Soc. Endocrinol.* **5**, 44–56.

THORSON, T. B. (1955). The relationship of water economy to terrestialism in amphibians. *Ecology*, **36**, 100–16.

THORSON, T. B. & SVIHLA, A. (1943). Correlation of the habitats of amphibians with their ability to survive the loss of body water. *Ecology*, **24**, 374–81.

INDUCED OVARIAN DEVELOPMENT IN DECAPITATED MOSQUITOES BY TRANSFUSION OF HAEMOLYMPH

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Ovarian development in mosquitoes appears to be under hormonal control. Evidence for this view has been derived from experiments involving removal of the endocrine organs assumed to be responsible for production of the hormone by decapitation or ligation of the thorax, before and after a critical period. (Detinova, 1945; Clements, 1956; Gillett, 1956*b*, 1957). Such evidence, however, even when supported by histological studies, is indirect and does not entirely exclude the possibility of alternative explanations; nor are the grounds for criticism removed when insects are joined in parabiosis (Wigglesworth, 1936, 1954; Williams, 1946). The present paper describes induction of ovarian development in mosquitoes, decapitated before the critical period, by transfusion of haemolymph from normal donors.

MATERIALS, METHODS AND TERMS

Mosquitoes

A strain of *Aëdes* (*Stegomyia*) *aegypti* (L.) originating from Lagos, Nigeria, and colonized in Entebbe for 10 years, was used in all the experiments. This strain was used also in previous experiments on the hormonal control of ovarian development (Gillett, 1956*b*, 1957). Each day female pupae were isolated in separate tubes within 5 hr. of pupation, by the 'dry' method described by Gillett (1956*a*); these were divided into three groups (test donors, test recipients and controls) and placed in an incubator at 28° C. Adults emerged 2 days later (day 0) in their separate tubes, and were fed on day 3.

Feeding. The period during which hormone is assumed to be released varies from mosquito to mosquito. This variation, however, can be decreased if the mosquitoes are not given fruit juice before the blood-meal (Gillett, 1957). It seems possible that previous water feeding may act in the same way as fruit feeding, the amount of water or fruit juice taken up by a mosquito being variable and equally difficult to control. In these experiments fruit feeding was excluded, and water feeding reduced to a minimum by the method of allowing adults to emerge on barely moist paper. Feeding, therefore, refers to blood meals only.

The mosquito's own blood, as distinct from ingested blood in the stomach, is referred to as haemolymph.

Treatment

In a previous experiment groups of fruit-starved mosquitoes reared from 'dry' pupae were decapitated at intervals after a feed, and were later dissected to see what proportion had developed their oocytes to maturity (Gillett, 1957). In the figure, which is based on these previous findings, it is shown that after 4 hr. the proportion rises steeply, and it is assumed that release of hormone usually occurs between 4 and 8 hr. after a feed. Nevertheless, it seemed possible that hormone, once released, might be rapidly inactivated, and thus that there might be a better chance of obtaining haemolymph with active hormone in the earlier half of this period, that is, between 4 and 6 hr. Similarly, it seemed possible that the oocytes might not enter a receptive state until shortly before, or at the time of, hormone release. Thus the optimal time for transfusion might be supposed to be between 4 and 6 hr. for the donors (50% of which might be expected to contribute hormone), and between 2 and 4 hr. for the recipients (8% of which might be expected to develop their oocytes to maturity independent of treatment). For convenience the period 4–6 hr. is referred to as the *active period*, and the period 2–4 hr. as the *receptive period*, although these two periods are likely to overlap in any individual mosquito.

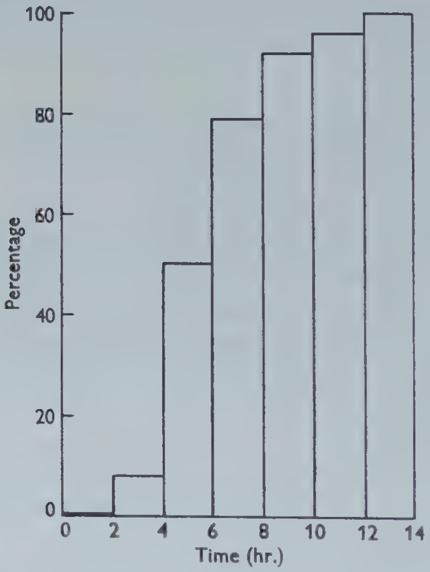

Fig. 1. Proportion of decapitated *A. aegypti* developing mature oocytes 48 hr. after a feed. Ordinate: percentage with mature oocytes (groups of 24). Abscissa: interval between feed and decapitation

Test mosquitoes were chilled for 100 sec. during the receptive period (the intervals between feeding and treatment being shown in each experiment) and decapitated, the wound being sealed with paraffin wax. About 0·2 mg. haemolymph was then withdrawn by thoracic puncture with a glass micropipette of 50–80 u

external diameter. Each of these *recipient* mosquitoes was then chilled again for 30 sec. and transfused through the same hole with about the same quantity of haemolymph, drawn from the thorax of a chilled *donor* mosquito, which had been fed 2 hr. before the recipient. Immediately after transfusion the donor was decapitated and the wound sealed with wax. Recipients and donors were then kept at 28° C. until day 5 (2 days after treatment), when both were sacrificed and dissected for ovarian inspection. The technique for thoracic puncture was very much simpler than that described by Shambaugh (1952) or by Weathersby (1952), although the dimensions of the micropipettes were based entirely on the figures given by Weathersby, which were confirmed by trial as being optimal. The technique was based on a method regularly used by Dr Loring Whitman of the Rockefeller Foundation Laboratories, New York, for the intrathoracic injection of mosquitoes, with virus.

Control mosquitoes were taken in pairs on the same day and at the same interval after being fed as were the test recipients in each trial. After chilling, each partner was decapitated and sealed with wax immediately before reciprocal transfusion. Thus each control mosquito became both donor and recipient, as the haemolymph was exchanged between pairs.

Response

For *A. aegypti* it has been shown that, whereas in mosquitoes decapitated before the critical period ovarian development stops at, or before Christophers's (1911) stage 3, in those decapitated after the critical period development goes on to maturity (stage 5). Thus there is presumptive evidence that the hormone is concerned only in development from stage 3 to maturity (Gillett, 1956*b*). Response to treatment, therefore, was regarded as positive only if mature oocytes were found when the mosquitoes were dissected on day 5.

The techniques used resulted in some premature deaths. In order to prevent any unconscious selection of data it was decided at the outset that each trial in each experiment should be continued until a predetermined number of mosquitoes had survived 48 hr. following treatment. Thus in Exp. 1 each of six trials was continued until 50 test recipients and 50 controls had survived for the requisite period, and Exp. 2 was continued until 100 test donor-recipient pairs had survived.

EXPERIMENTS

Two main experiments were carried out, both being based on the assumption that hormone produced in the head (presumably from the neurosecretory cells of the brain) either exerts its effect by being released directly into the haemolymph, or by activating the corpus cardiacum or corpus allatum which are situated in the thorax (Bodenstein, 1945; Scharrer, 1952, Thomsen, 1952; Gillett, 1956*b*). The plan was to see if haemolymph from donors, presumed to contain hormone, would induce ovarian development to maturity when transfused to mosquitoes deprived of the capacity to produce their own hormone. It was clear, however, that by taking recipients and controls in the receptive period, some of them would in any case develop oocytes to maturity; the question, therefore, was whether or not the response rate would be higher in test recipients than in the controls.

Experiment 1. As the best time for treatment was not known, it was decided first to take recipients early in the receptive period and to transfuse them with haemolymph from donors early in the active period; then to take recipients late in the receptive period and to transfuse them from donors late in the active period. Having thus straddled the target, intermediate times were chosen in a series of trials in the hope of finding the optimal time both for recipients and donors.

Table 1. *Effect of haemolymph transfusion on development of the ovaries in decapitated* Aëdes aegypti

Trial no.	Test mosquitoes			Control mosquitoes		
	Interval (hr.)* between feed and transfusion		Proportion of recipients with mature oocytes 48 hr. after feed	Interval (hr.)* between feed and transfusion		Proportion of recipients with mature oocytes 48 hr. after feed
	Donor	Recipient		Donor	Recipient	
1	4	2	4/50	2	2	3/50
2	$4\frac{1}{2}$	$2\frac{1}{2}$	14/50	$2\frac{1}{2}$	$2\frac{1}{2}$	5/50
3	$4\frac{1}{2}$	$2\frac{1}{2}$	9/50	$2\frac{1}{2}$	$2\frac{1}{2}$	6/50
4	$5\frac{1}{4}$	$3\frac{1}{4}$	9/50	$3\frac{1}{4}$	$3\frac{1}{4}$	6/50
5	$5\frac{1}{2}$	$3\frac{1}{2}$	9/50	$3\frac{1}{2}$	$3\frac{1}{2}$	4/50
6	6	4	20/50	4	4	17/50
Total			65/300			41/300

* $\pm 2\frac{1}{2}$ min.

The results, arranged in order of increasing time interval between feeding and treatment, are shown in Table 1. It will be noted that the time interval in trials 2 and 3 was the same. The reason for this was that the results of trial 2 showed a difference in response rate between test recipients and controls which was significant at the 2·5% level (χ^2 for 1 degree of freedom $= 5{\cdot}263$, $0{\cdot}02 < P < 0{\cdot}025$), and it was felt that if this result was attributable to the treatment, then it should be reproducible. The results of trial 3, however, showed less difference in response rate between test recipients and controls, and, in fact, in none of the trials which followed was such a difference recorded. Nevertheless, in all six trials the difference was in the expected direction, a result which is significant at the 1% level in a one-sided test concerning increase and not merely change in response rate (t for 5 degrees of freedom $= 3{\cdot}554$, $0{\cdot}005 < P < 0{\cdot}01$). The over-all picture is, of course, largely influenced by the result of trial 2, which increases the mean difference in response rate, but at the same time increases the standard deviation. If this trial is excluded the results are significant at the 0·5% level for a one-sided test (t for 4 degrees of freedom $= 4{\cdot}743$, $0{\cdot}001 < P < 0{\cdot}005$). It seems, therefore, that transfusion of haemolymph to recipients between 2 and 4 hr. after a feed, from donors fed 2 hr. earlier, results in an increase in response rate over those transfused by donors fed at the same time.

So far consideration has been given to difference in response rate in test recipients and controls, irrespective of the fate of the oocytes in the donors. Actually, these donors were decapitated immediately after contributing haemolymph in order to see if they had, in fact, entered the period of hormone production by the time treatment was given. But while each trial was continued until 50 recipients and 50 controls had survived, no such precautions were necessary with the donors; if a recipient survived for 48 hr. following treatment, the result was scored whether the donor survived or not; if, on the other hand, a recipient died prematurely, a second recipient was transfused by a second donor, no matter what the fate of the first donor happened to be. Thus, while all recipients and controls had a denominator of 50 in each trial, the number of surviving donors that could be scored varied from trial to trial. The results are given in Table 2 and show a steady increase in the proportion of donors which went on to develop mature oocytes from 38% at 4 hr. to 80% at 6 hr.

Table 2. *Effect of haemolymph withdrawal and decapitation on development of the ovaries in* Aëdes aegypti

Trial no.	Interval (hr.)* between feed and decapitation	Proportion of mosquitoes with mature oocytes 48 hr. after feed
1	4	11/29 (38%)
2	4½	35/62 (57%)
3	4½	25/43 (58%)
4	5¼	20/34 (59%)
5	5½	24/33 (73%)
6	6	28/35 (80%)
Total		143/236 (61%)

* ± 2½ min.

Experiment 2. About one-quarter of all donors and recipients failed to survive treatment. It followed, therefore, that the chance of the two partners in a single donor-recipient pair both surviving was only about one in two. Nevertheless, it was decided to study the response rate in donor-recipient pairs, discarding all results except when donor and recipient both survived. The question, then, was whether the response rate in recipients of haemolymph from positive donors was higher than that in recipients of haemolymph from negative donors.

As it was essential to have negative as well as positive donors it was decided to take mosquitoes at 4½ hr. after a feed (see Table 2), and to transfuse their haemolymph to decapitated recipients at 2½ hr. after a feed. The choice of these time intervals not only ensured that there would be a large number of negative donors, but allowed all surviving donor-recipient pairs from trials 2 and 3 of Exp. 1 to be incorporated into the results. All donors were decapitated immediately after treatment. The results, given in Table 3, do not show a significant difference in response rate between recipients of haemolymph from positive donors and those from negative donors, nevertheless, the difference is again in the expected direction, the response rate of one being double that of the other.

Table 3. *Effect of haemolymph transfusion from positive and negative donors on development of the ovaries in decapitated* Aëdes aegypti.

Proportion of recipients developing mature oocytes following transfusion		Total
From positive donors	From negative donors	
14/63 (22 %)	4/37 (11 %)	18/100
χ^2 for 1 degree of freedom = 1·356*, 0·10 < P < 0·125 (for one-sided test)		

* Yates's correction applied.

DISCUSSION

The use of transfusion methods to demonstrate the presence of hormones in the haemolymph of insects has met with varying success (Fraenkel, 1935; Fukuda, 1938; Mokia, 1941; Meyer, 1953; Hasegawa, 1957). In theory it would appear to offer the final proof that hormonal mechanisms are involved, but in practice there may be too many unknown factors to allow clear cut results.

In the mosquito *A. aegypti* it has been assumed, as a working hypothesis, that some time between 4 and 6 hr. after a feed hormone released from the head (presumably from the neurosecretory cells of the brain) is present in the haemolymph, and normally results in the development of activated oocytes from stage 3 to maturity. Thus, if haemolymph is withdrawn from a donor mosquito during this period and transfused to a mosquito which has been deprived of the capacity to produce its own hormone, normal development of the ovaries might be expected to follow in the recipient. It is not known, however, whether hormone, once released, remains free for some time in the haemolymph, or whether, even if produced over a long period, it is inactivated almost as fast as it is produced. Nor is it known whether the receptor organs (for example, other endocrine organs or perhaps the oocytes themselves) enter a receptive period of limited duration, or whether they require a constant supply of freshly liberated hormone over a prolonged period.

The chances, then, of taking haemolymph from a donor at the optimal time and transfusing it into a recipient also at the optimal time might be very slender indeed. Better results might have followed had repeated transfusions been given to each recipient. This possibility was realized at the time, but it was felt that mosquitoes are not sufficiently robust to withstand such treatment. Better results might also have been obtained had donors and recipients been fed at the same time; thus while the recipients would have been decapitated between 2 and 4 hr. after the feed, transfusion would have been postponed for a further 2 hr. This was, in fact, tried, but as it necessitated extra manipulation it was abandoned in favour of the technique described.

Besides these and other unknown physiological factors, there exist possible indirect effects of the necessary manipulative treatment. That chilling and the act of decapitation itself do not adversely affect the response seems clear from the results shown in Table 2 and those of many other experiments on this species. One must, however, consider the possible effects of puncture and of temporary reduction in haemolymph volume between withdrawal and replacement by fresh haemolymph. In these experiments haemolymph withdrawn from donors was not replaced, yet 61% (143/246) developed oocytes to maturity after decapitation (Table 2). This is not significantly different from the proportion of 12/24 obtained previously by Gillett (1957) following simple decapitation between 4 and 6 hr. after a feed (χ^2 for 1 degree of freedom = 1·015, $0·30 < P < 0·50$).

It is well known that changes occur in haemolymph on exposure to air, which are manifested by darkening (Dennell, 1947; Fraenkel & Rudall, 1947). This change takes 90–120 sec. in *A. aegypti*, but as the interval between withdrawal of haemolymph from a donor to transfusion to a recipient was only 20–40 sec. this change did not occur in practice. The amount of haemolymph exchanged was 0·2 mg., but as the average weight of unfed *A. aegypti* is only 1·32 mg. (Roy, 1936), this represents a substantial proportion (15%) of the body weight.*

That apparently minor differences in technique may have far-reaching effects on the results is shown by the difference in response between fruit-fed and fruit-starved mosquitoes, and emphasizes the need for caution when comparing results.

It has been shown that while initiation of ovarian development in *A. aegypti* is apparently independent of endocrine organs in the head (Clements, 1956; Gillett, 1956*b*), development stops at or before stage 3 unless the head is left intact for a period of 4–8 hr. following the feed (Gillett, 1956*b*, 1957). If, however, groups of fed normal *A. aegypti*, kept at 28° C., are sacrificed and dissected at hourly intervals, it is found that further development of stage 3 oocytes does not occur until about 14 hr. after the feed. This perhaps supports the possibility, which has already been mentioned, that the hormone from the head serves in turn to activate other endocrine organs, which must lie posterior to the head (presumably in the thorax). While preliminary trials involving decapitation and extirpation of the anterior part of the thorax, and delayed transfusion between 8 and 14 hr. after the feed, have not so far supported this, it is hoped to be able to investigate the possibility further.

SUMMARY

1. The final stages of ovarian development in the mosquito *Aëdes aegypti* appear to be under the control of a hormone which is produced in the head.

2. Decapitation of groups of mosquitoes before a critical period prevents final development of the ovaries; decapitation after the critical period allows development of the ovaries to maturity.

3. Transfusion of haemolymph from donor mosquitoes in the middle of the critical period to recipient mosquitoes decapitated at a stage just before, or very early in the critical period, results in a higher number developing their oocytes to

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* Mellanby (1939) has stated that the amount of haemolymph in insects varies from 15 to 70% of the total body weight.

maturity than when transfusion is between donors and recipients at the same stage.

4. Similarly, transfusion from known positive donors (i.e. those which by decapitation and subsequent dissection were known to have already produced hormone at the time of transfusion) results in a higher number of recipients developing oocytes to maturity, than when transfusion is from known negative donors.

I am greatly indebted to Dr C. L. Chiang, Assistant Professor of Biostatistics, University of California, and to Dr Loring Whitman of the Rockefeller Foundation Laboratories, New York: the former for a most helpful discussion of the results and for pointing out the use of the one-sided test for handling the figures; and the latter for the privilege of seeing his simplified microinjection technique at first hand during a Rockefeller Foundation Fellowship in 1955–56.

REFERENCES

BODENSTEIN, D., (1945). The corpora allata of mosquitoes. *Bull. Conn. Agric. Exp. Sta.* no. 488, pp. 396–405.

CHRISTOPHERS, S. R. (1911). The development of the egg follicle in anophelines. *Paludism*, **2**, 73–88.

CLEMENTS, A. N. (1956). Hormonal control of ovary development in mosquitoes. *J. Exp. Biol.* **33**, 211–23.

DENNELL, R. (1947). A study of an insect cuticle: the formation of the puparium of *Sarcophaga falculata* Pand. (Diptera). *Proc. Roy. Soc.* B, **134**, 79–110.

DETINOVA, T. S. (1945). On the influence of glands of internal secretion upon the ripening of the gonads and the imaginal diapause in *Anopheles maculipennis*. (In Russian with English summary.). *Zool. Zh.* **24**, 291–8.

FRAENKEL, G. (1935). A hormone causing pupation in the blowfly *Calliphora erythrocephala*. *Proc. Roy. Soc.* B, **118**, 1–12.

FRAENKEL, G. & RUDALL, K. M. (1947). The structure of insect cuticles. *Proc. Roy. Soc.* B, **134**, 111–43.

FUKUDA, S. (1938). Note preliminaire sur la transformation de la peau larvaire en puparium chez les larves de *Sturmia sericariae*. *Proc. Imp. Acad. Japan*, **13**, 114–16.

GILLETT, J. D. (1956*a*). Genetic differences affecting egg-laying in the mosquito *Aëdes* (*Stegomyia*) *aegypti* (Linnaeus). *Ann. Trop. Med. Parasit.* **50**, 362–74.

GILLETT, J. D. (1956*b*). Initiation and promotion of ovarian development in the mosquito *Aëdes* (*Stegomyia*) *aegypti* (Linnaeus). *Ann. Trop. Med. Parasit.* **50**, 375–80.

GILLETT, J. D. (1957). Variation in the time of release of the ovarian development hormone in *Aëdes aegypti*. *Nature, Lond.*, **180**, 656–7.

HASEGAWA, K. (1957). The diapause hormone of the silkworm, *Bombyx mori*. *Nature, Lond.*, **179**, 1300–1.

MELLANBY, K. (1939). The function of insect blood. *Biol. Rev.* **14**, 243–60.

MEYER, J. H. (1953). Die Bluttransfusion als Mittel zur Überwindung letaler Keimkombination bei Lepidopteren-Bastarden. *Z. wien. ent. Ges.* **38**, 41–80.

MOKIA, G. G. (1941). Contribution to the study of hormones in insects. *C.R.* (*Doklady*) *Sci. U.R.S.S.* **30**, 371–3.

ROY, D. N. (1936). On the role of blood in ovulation in *Aëdes aegypti* Linn. *Bull. Ent. Res.* **27**, 423–9.

SHAMBAUGH, G. H. (1952). Microinjection technique in mosquitoes. *Mosquito News*, **12**, 216.

SHARRER, B. (1952). Neurosecretion. XI. The effects of nerve section on the intercerebralis-cardiacum-allatum system of the insect *Leucophaea maderae*. *Biol. Bull., Woods Hole*, **102**, 261–72.

THOMSEN, E. (1952). Functional significance of the neuro-secretory brain cells and the corpus cardiacum in the female blow-fly, *Calliphora erythrocephala* Meig. *J. Exp. Biol.* **29**, 137–72.

WEATHERSBY, A. B. (1952). The role of the stomach wall in the exogenous development of *Plasmodium gallinaceum* as studied by means of haemocoel injection of susceptible and refractory mosquitoes. *J. Infect. Dis.* **91**, 198–205.

WIGGLESWORTH, V. B. (1936). The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.* **79**, 92–121.

WIGGLESWORTH, V. B. (1954). *The Physiology of Insect Metamorphosis.* Monographs in Exp. Biol. No. 1. Cambridge University Press.

WILLIAMS, C. M. (1946). Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull., Woods Hole*, **90**, 234–43.

THE MIGRATION OF SPERMATOZOA IN THE FEMALE OF *RHODNIUS PROLIXUS* STAL.

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INTRODUCTION

In *Rhodnius*, as in many insects, a spermatophore containing the semen is deposited in the bursa copulatrix of the female during copulation, and the spermatozoa then migrate to the spermathecae where they are stored until they are used in fertilization. In some insects this migration may be very complex, as in the Lepidoptera (Musgreave, 1937; and many others), and in seeking to explain these movements many authors have assumed that the spermatozoa play an active part and some have invoked chemotaxis (Heberdey, 1931). Evidence to be presented in this paper will demonstrate that the migration in *Rhodnius* is a result of contractions in the female ducts set up by a secretion from the male acting through a peripheral nervous system and that the spermatozoa play no active part in the migration.

The spermatophore of *Rhodnius* has been described by Khalifa (1950*a*), and its mode of formation and the events at copulation will be examined in a later paper. All that concerns us here is that the spermatophore is placed in the bursa copulatrix so that it fills the bursa and the slit containing the semen encloses the lip-like vestibulum (see Fig. 1). By killing the females at various times after copulation, and sectioning the reproductive ducts, it was ascertained that the spermatozoa travelled to the spermathecae via the lumen of the common oviduct and that they began to arrive at the spermathecae within 5–10 min after the termination of mating. There was no particular orientation of the spermatozoa in the oviduct or spermathecae.

THE OPAQUE ACCESSORY SECRETION

In the male reproductive system of *Rhodnius* there are accessory glands consisting of four finger-like lobes on each side. Three of the four lobes contain a transparent, viscous material which forms the spermatophore; this material is of little importance in this study. The fourth lobe, containing an opaque, granular secretion, is responsible for the movements of the spermatozoa in the female. If both of the opaque glands are removed from a male, and if, after a recovery period of about a week, the male (deprived of its opaque glands) is mated to a virgin female, a normal spermatophore will be produced; but the spermatozoa, also apparently normal, will not leave the bursa. The spermathecae are found to be empty when the female is dissected 5 hr. after copulation. If, on the other hand, the male is operated on one side only, the spermatozoa ascend the oviduct to the spermathecae in the normal way.

In an attempt to ascertain the site of action of the secretion, the opaque accessory glands of several males were injected with dyes and these males were mated to females which were dissected immediately after copulation to see whether the dye could be detected. Only twice did the dye (in both cases methylene blue) appear; in each case it was found between the dorsal wall of the bursa and the spermatophore just posterior to the mass of semen and the vestibulum. A more convincing observation is that a faint cloudiness just posterior to the slit can be discerned on the dorsal surface of the normal spermatophore, and that this cloudiness is absent from spermatophores produced by males which have been deprived of their opaque glands. Nonidez (1920) suggested that a secretion in *Drosophila* may activate the spermatozoa, causing them to swim into the spermathecae. The opaque secretion in *Rhodnius* does not appear to function in this way, for in spermatophores produced by males from which the seminal vesicles have been removed (i.e. spermatophores without semen), the opaque secretion is confined to the area posterior to the slit which would normally contain the semen.

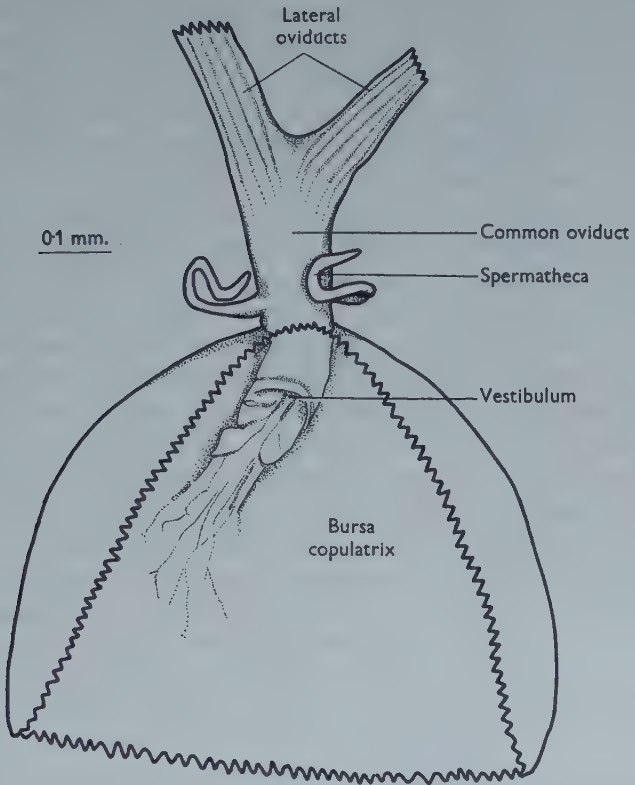

Fig. 1. Ventral view of the female reproductive ducts of *Rhodnius*. The cement gland opening into the bursa has been removed and the floor of the bursa has been slit.

EFFECT OF THE OPAQUE SECRETION ON THE FEMALE ORGANS

In females which are carrying spermatophores certain rhythmic contractions of the oviduct can be detected if the female is opened under insect Ringer. These movements take the form of peristaltic contractions beginning with a sharp constriction just above the spermathecae which travels downwards along the oviduct and spreads out over the muscular dorsal surface of the bursa copulatrix. Each wave of peristalis is accompanied by a longitudinal contraction of the oviduct resulting in that organ being thrust down into the mass of semen held in place by the spermatophore. These contractions continue at a frequency of about four per minute until the preparation dies, usually a matter of 2 or 3 hr. The same contractions have been noted occasionally in females without spermatophores, but they did not persist for more than a minute or two.

In order to test whether a relationship existed between the contractions and the opaque secretion, some of this material was placed in the bursa in the following manner. A flap of cuticle was removed from the last sternite to reveal the membranous floor of the bursa, which was cut, the cut edges being reflected over the cut edges of the cuticle to keep blood out of the bursa. An opaque accessory gland was placed in the bursa and its wall ruptured so as to spill out the contents. Under such circumstances the peristaltic contractions previously described began and persisted until the bursa was rinsed with insect Ringer or the preparation died. After rinsing, the frequency of the contractions gradually declined until, after 15–30 min., the contractions ceased. Persistent contractions were not initiated by the transparent accessory secretion from the male, by fat body, or by insect blood.

Further, the same activation by the opaque secretion occurred in preparations obtained by severing the lateral oviducts and the nerves and tracheae leading to the genital organs and gently pulling the bursa away from the ventral body wall. The preparation, consisting of bursa, common oviduct, and part of the lateral oviducts, was pinned out ventral side up in a drop of Ringer and the bursa was slit open to reveal the vestibulum. This operative procedure often initiated peristaltic contractions which were sometimes quite persistent, but normally they ceased or their frequency fell below two per minute within an hour or two. The application of the opaque secretion either initiated contractions in the quiet preparations or increased their frequency from less than two to five or six per minute in those which were undergoing 'spontaneous' contractions. Transparent secretion, fat body, and insect blood failed to induce persistent contractions in these isolated preparations. It must be pointed out that the preparation is capricious and that considerable patience is required to demonstrate the effect. There can be no doubt that the phenomenon exists; it has been demonstrated in more than fifty preparations.

THE MODE OF ACTION OF THE OPAQUE SECRETION

The following observations suggested that the opaque secretion was acting through the nervous system rather than directly on the muscle. The contractions began at the level of the spermathecae some distance from the point of application of the stimulus in the bursa, indicating that some form of conduction is involved. Moreover, the muscular pad surrounding the vestibulum is the only area in the bursa sensitive to the secretion; cautery of the pad with a loop of electrically heated wire stopped the contractions, whereas cautery or excision of the ventral or lateral walls of the bursa did not interfere with the effect. Application of the opaque secretion to any part of the outside of the intact bursa failed to elicit a response.

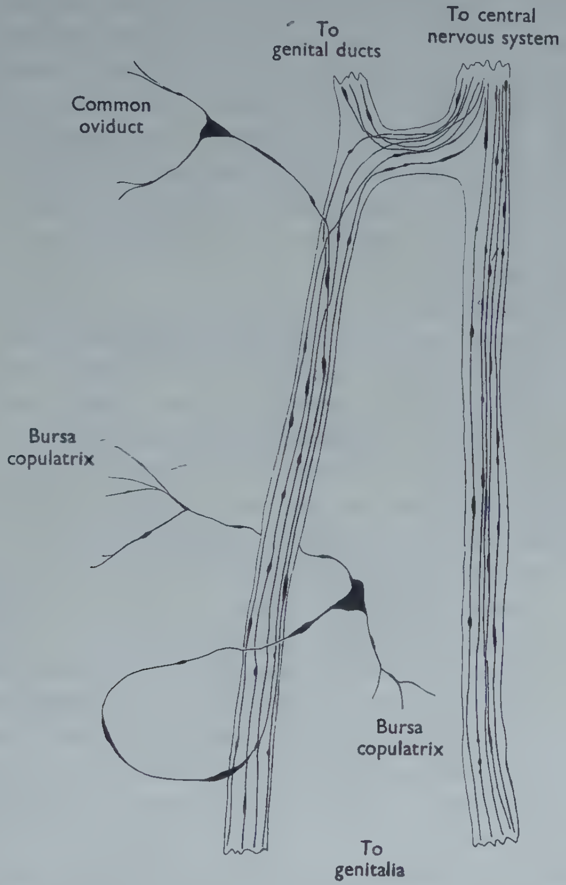

Fig. 2. Diagram of the nerves connecting the bursa and the oviduct as revealed by methylene blue staining in the female of *Rhodnius*.

Staining by injection of methylene blue into the living animal revealed a direct nervous connexion between the dorsal part of the bursa copulatrix and the common oviduct in a number of whole mount preparations of the genital ducts. Fig. 2 is a diagram showing one of these connexions. Fine branches from the muscular pad in the bursa unite in a nerve fibre which travels anteriorly in one of the bundles of nerves supplying the genitalia. At the level of the spermathecae it leaves this bundle and ends in the muscles of the common oviduct. Only one or two such connexions were stained in any one preparation. From the physiological evidence one might expect to find ganglion cells in this system, but no structures that could be positively identified as nerve cell bodies were seen.

THE MECHANISM IN THE INTACT INSECT

The foregoing constitutes evidence for a possible mechanism by which the spermatozoa may move into the spermathecae, but is there any evidence that such a mechanism operates in the intact insect? The motility of *Rhodnius* spermatozoa is unaffected by the oxygen deprivation resulting from exposure to nitrogen, but muscular paralysis results under such conditions. Mating pairs of *Rhodnius*, the females of which were previously unmated, were exposed to nitrogen 5 min. before mating normally ended (i.e. 25 min. after its initiation). The pairs usually separated and paralysis ensued within 3 min. The nitrogen was passed slowly over the insects for 5 hr., after which time the females were dissected quickly and the spermathecae examined. Only the results of experiments in which the male subsequently recovered were considered.

Of ten such experiments, the females in eight instances contained no semen in the spermathecae; in the other two it was present in greatly reduced amounts. Normally, of course, the spermathecae would be packed with spermatozoa 5 hr. after mating. Controls consisted of five pairs which were treated as before, but were allowed to recover and after five additional hours were dissected. There were large quantities of spermatozoa in the spermathecae of all five females. Paralysis of the muscles of the intact insect with nitrogen prevents the ascent of the spermatozoa.

These results, however, do not exclude the possibility that the movements of the spermatozoa are partly responsible for the ascent. It was therefore desirable to devise a method for placing motionless spermatozoa in the spermatophore without interfering with the movements of the oviduct. The following technique met with some success. A previously unmated female was mated and the pair gassed as described above. After half an hour the female was operated upon as quickly as possible in the manner to be described. A small piece of cuticle was removed from the ventral posterior abdomen to expose the transparent floor of the bursa through which the subsequent manipulations could be observed. An injection needle mounted on a micromanipulator was inserted through the genital opening and pushed through the spermatophore until the tip of the needle entered the centre of the mass of semen. A 0·01 M solution of cetyl trimethyl ammonium bromide (CTAB) in insect Ringer containing a little methylene blue to act as a tracer was injected. CTAB, a surface active agent, is a powerful spermicide but requires some time to act (Koefoed-Johnsen & Mann, 1954). It was therefore necessary to return the female to nitrogen for 2 hr. In the concentrations used, CTAB had no apparent effect on other tissues, but it was necessary to buffer the solution to pH 5·6 to prevent the liquefaction of the spermatophore.

After this 2 hr. period under nitrogen anaesthesia the female was returned to air to recover and 5 hr. later was sacrificed. If the spermatozoa in the bursa were inactive, the spermathecae were examined for the presence of spermatozoa. Out of twenty matings, there were only seven in which most of the spermatozoa in the bursa were inactive. In every case there was semen in the spermathecae, but in only three of these were all the spermatozoa in the spermathecae inactive. In the other four a small proportion of the spermatozoa were active, but most of them were quite motionless. Activity of the spermatozoa is not a prerequisite for the ascent.

The opaque secretion will activate the isolated oviducts; but is some degree of nervous control necessary for the ascent in the intact animal? In *Rhodnius* there are no central ganglia in the abdomen, so that removal of the abdomen isolates it from the central nervous system. The abdomen of females from mating pairs which had been anaesthetized with nitrogen as before were removed by ligation and placed in air to recover. After 5–7 hr. the abdomens were dissected and the spermathecae examined. Only those abdomens which were judged to be alive (i.e. gut, heart and oviduct capable of contraction) were included in the results of these experiments. Since the spermathecae in all five such abdomens were packed with spermatozoa, nervous control is not necessary for the ascent of the spermatozoa.

The fact that the spermatozoa move up the oviduct while the contractions move down presents some difficulty, and the following is a suggested explanation. At the same time as the oviduct is contracting longitudinally and thrusting the vestibulum down into the mass of semen, the lip of the vestibulum is caused to gape and then close firmly by the peristalsis. In this way the vestibulum makes bite-like motions in the mass of semen. The peristalsis spreading over the surface of the bursa exerts pressure on the spermatophore which prevents each 'bite' from moving backwards. Successive 'bites' must then push the semen up the oviduct. The spermatozoa are prevented from moving beyond the level of the spermathecae in the oviduct by constrictions which form there at the beginning of every contraction and probably also by hair-like projections of the intima in that region.

The spermathecae are blind tubes and the movement of the spermatozoa into them requires either that they be stretched or that some of the secretion which they contain be displaced. The spermathecae of recently mated females appear to be no longer than those of virgin females. However, the spermathecae twitch continuously while the oviduct is contracting, and it is suggested that during these twitchings the lumen of the spermatheca expands momentarily allowing the spermatozoa to enter. When the lumen returns to its normal state, the spermatozoa are prevented from returning to the oviduct by a series of teeth, projections of the intima in the proximal portion of the spermatheca. These teeth act as a sieve, permitting the passage of the secretion and thereby equalizing the pressure. That the spermatozoa are pushed into the spermathecae gains support from the following experiment. The spermathecae of several females were removed and the females were mated to normal males. Upon dissection 5 hr. after mating, masses of spermatozoa were seen projecting from the small holes left by removal of the spermathecae. The spermatozoa had evidently been forced out of these tiny holes by pressure from within the oviduct.

THE MECHANISM IN OTHER INSECTS

In *Rhodnius*, then, the migration of the spermatozoa takes place as a result of contractions set up in the oviduct by the opaque accessory secretion from the male acting through a nervous system. Is there any evidence that a similar mechanism operates in other insects? Of course a number of insects do not produce a spermatophore, but transfer the semen direct to the spermathecae as in *Oncopeltus* (Bonhag & Wick, 1953). Khalifa (1949*a*) has described the spermatophore in *Gryllus* and demonstrated that the migration is brought about by pressure from within the spermatophore, but this type of sperm transfer appears to be unique among the insects. For other species with more typical spermatophores there is in the literature a considerable body of circumstantial evidence that a mechanism similar to the one described for *Rhodnius* may exist. The brief survey to follow presents some of this evidence.

Khalifa (1950*b*) mentions a milky granular secretion associated with the spermatophore of the cockroaches *Blatella* and *Periplaneta*. In *Periplaneta* the milky secretion originates in the complex accessory glands of the male (Gupta, 1947), and in the present study it was found that a suspension of these glands in insect Ringer would activate the oviducts of *Rhodnius* in the same way as the opaque secretion from the *Rhodnius* male. Similarly, the opaque secretions of the reduviid, *Triatoma infestans* causes contractions in the oviduct of *Rhodnius*. Khalifa (1949*b*) mentions a granular secretion in the semen of Trichoptera and describes powerful contractions in the bursa of Neuroptera which also possess an opaque secretion.

Several authors (Omura, 1938; Musgreave, 1937; Norris, 1932) have suggested that the migration of the spermatozoa along a complicated route from the bursa to the receptaculum seminis in Lepidoptera may be a result of contractions in the ducts involved, and Hewer (1934) has seen spermatozoa being propelled in this way along the ductus seminalis of *Zygaena*.

In the Coleoptera, Khalifa (1948) has suggested that the spermatophore in *Coccinella* is emptied by muscular contractions in the bursa and has observed a granular secretion between the spermatophore and the wall of the bursa. Preliminary observations on *Tenebrio molitor* in the present work have revealed rhythmic contractions of the ducts of females carrying spermatophores; these contractions are absent in unmated females. A granular secretion is present in the semen.

SUMMARY

1. Removal of the opaque accessory glands from a male *Rhodnius* prevents the normal migration of the spermatozoa in a female which is inseminated by it.
2. The opaque accessory secretion induces rhythmic contractions in the oviducts, probably by acting through a peripheral nervous system.
3. Paralysis of the muscles of the female without interfering with the motility of the spermatozoa prevents the ascent of the spermatozoa.
4. Killing the spermatozoa in the bursa has no effect on their subsequent migration.

5. The central nervous system is not essential for the migration.
6. It is concluded that the migration of spermatozoa in the female of *Rhodnius proxlixus* is a result of rhythmic contractions set up in the oviduct by the opaque accessory secretion of the male acting through a peripheral nervous system. Evidence in the literature suggests that the mechanism may operate in a number of spermatophore-forming species.

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REFERENCES

Bonhag, P. F. & Wick, J. R. (1953). The functional anatomy of the male and female reproductive systems of the milkweed bug. *J. Morph.* **93**, 171–284.

Gupta, P. O. (1947). On the structure and formation of the spermatophore in the cockroach *Periplaneta americana*. *Ind. J. Ent.* **8**, 79–84.

Heberdey, R. F. (1931). Zur Entwicklungsgeschichte vergleichenden Anatomie und Physiologie der weiblichen Geschlechtsausfurwege der Insekten. *Z. Morph. Ökol. Tiere*, **22**, 416–586.

Hewer, H. R. (1934). Studies in *Zygaena*. Part II. The mechanism of copulation and the passage of sperm in the female. *Proc. Zool. Soc. Lond.* **2**, 513–27.

Khalifa, A. (1948). The biology of spermatophore production in insects. Thesis, Cambridge.

Khalifa, A. (1949*a*). The mechanism of insemination and the mode of action of the spermatophore in *Gryllus domesticus*. *Quart. J. Micr. Sci.* **90**, 281–292.

Khalifa, A. (1949*b*). Spermatophore production in Trichoptera and some other insects. *Trans. R. Ent. Soc. Lond.* **100**, 449–71.

Khalifa, A. (1950*a*). Spermatophore production and egg-laying behaviour in *Rhodnius prolixus*. *Parasitology*, **40**, 283–9.

Khalifa, A. (1950*b*). Spermatophore production in *Blattella germanica*. *Proc. R. Ent. Soc. Lond.* A, **25**, 53–61.

Koefoed-Johnsen, H. H. & Mann, T. (1954). Studies on the metabolism of semen. 9. Effect of surface active agents with special reference to the oxidation of succinate by spermatozoa. *Biochem. J.* **57**, 406–10.

Musgreave, A. J. (1937). Histology of the male and female reproductive organs of *Ephestia kuhniella* Zeller (Lepidoptera). I. The young imagines. *Proc. Zool. Soc. Lond.* **107** B, 337–64.

Norris, M. J. (1932). Contributions toward the study of insect fertility. I. The structure and operation of the reproductive organs of the genera *Ephestia* and *Plodia*. *Proc. Zool. Soc. Lond.* **102**, 595–611.

Nonidez, J. G. (1920). Internal phenomena of reproduction in *Drosophila*. *Biol. Bull. Woods Hole*, **39**, 210–30.

Omura, S. (1938). Structure and function of the female genital system of *Bombyx mori*, with special reference to the mechanism of fertilization. *J. Fac. Agric. Hokkaido Univ.* **40**, 111–28.

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